

# EXHIBIT 21

## PART I

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KABAT and MAYER'S  
**EXPERIMENTAL  
IMMUNOCHEMISTRY**

*Revised and Enlarged*

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*With Chapters on*

*Complement and Complement Fixation  
and  
Kjeldahl Nitrogen Determination*

by

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## Chapter 4

### Complement and Complement Fixation

THE TERM complement refers to a group of five serum factors, constituting a cytotoxic reaction system, which plays a role in various immunological reactions. In conjunction with the appropriate antibodies, or other "sensitizing agents", complement kills certain susceptible bacteria and protozoa, lyses erythrocytes, immobilizes treponemes, promotes immune-adherence reactions (1-9), and phagocytosis (10), and in some cases participates in the neutralization of viruses (11, 231). Recent studies have also focused attention on the possible role of complement in hypersensitivity reactions (12-15). In general, complement, if present, and under appropriate experimental conditions, enters into combination and reacts with many kinds of antigen-antibody complexes. Muir (16) has defined complement as "that labile substance which is taken up by antigen-antibody aggregates." However, since the capacity to combine with antigen-antibody complexes is not an exclusive property of complement, but is shared by other serum constituents, for example, rheumatoid factor (17) and conglutinin (18), Muir's definition is too general and should be restricted by incorporation of the function of complement.

Complement has been reviewed recently by Schmidt (18). Reference should be made also to earlier reviews by Osborne (19), Pillemer (20), Doerr (21), Heidelberger and Mayer (22) and Mayer (23). A recent review of complement fixation has been presented by Osler (24).

The discovery of complement emerged from observations in the 1880's by Buchner (25), von Fodor (26), and Nuttall (27) that blood serum exerts a destructive influence

upon bacteria. Nuttall (28) found that this bactericidal power decreases as the serum ages and is lost rapidly on heating at 56°C. Following Pfeiffer and Issaef's (29) discovery of the lysis of cholera vibrio injected into the peritoneal cavity of immune guinea pigs, Bordet (30) demonstrated bacteriolysis *in vitro* and also showed that the bacteriolytic power of immune serum, when destroyed by heating to 56°C., can be restored by addition of normal serum, establishing that two distinct substances are required for bacteriolysis. One, termed complement by Bordet and considered identical with Buchner's alexin, is present in normal serum, and can be readily destroyed by heat. The other, a more stable factor termed the sensitizing substance, bacteriolysin or bactericidin, is an antibody, which is produced, or increased, as a result of immunization.

Later, it was found that specific hemolysis also involves two factors, namely, complement and antibody to erythrocytes. This antibody is referred to as hemolysin, amboceptor, hemolytic antibody or hemolytic sensitizer. Ehrlich and Morgenroth (31) showed that the hemolysin or amboceptor can combine with homologous erythrocytes in the presence or absence of complement, and that sufficient amounts of hemolysin will cause hemagglutination in the absence of complement. However, uptake of complement by erythrocytes takes place only if amboceptor is present, and furthermore, hemolysis will ensue only if experimental conditions are suitable for the destructive action of complement. The various factors which influence the hemolytic activity of complement will be discussed in detail in later sections.

These early experiments on specific immune bacteriolysis and hemolysis paved the way for numerous and diverse studies on the role of complement in humoral and cellular immunity, as well as its participation in certain allergic reactions. The problems associated with complement are far from settled and controversies on its nature rage today as they did in the days of Ehrlich and Bordet. The difficulties which have plagued this branch of immunology are largely attributable to its complexity, for complement is not a single substance, but comprises at least five components, designated C'1, C'2, C'3a, C'3b and C'4. The sequence of action of these factors has been established, but the nature of each of the constituent reaction steps has not yet been elucidated. Furthermore, these factors are recognized only in terms of the effects they produce; little is known about their chemical nature.

In addition to its capacity to react with sensitized bacteria, protozoa or cells from higher organisms, complement will combine with antigen-antibody complexes made from soluble antigens; indeed, the various immunological activities of complement may be regarded as consequences of its capacity to combine and react with antigen-antibody complexes. If antigen-antibody aggregates are formed in fresh serum containing complement, or, if previously formed washed aggregates are added to fresh serum, the hemolytic complement activity for sensitized red cells (red cells plus hemolytic antibody) disappears. In the language of the immunologists, the complement has been "fixed" by the antigen-antibody complexes. This is the basis for the complement fixation test devised by Bordet and Gengou (32), which is used widely in diagnostic serology. The sensitivity of this test is great, about 0.05 to 0.1  $\mu$ g of antibody nitrogen being sufficient for a positive reaction. This is approximately one-tenth the minimal quantity of antibody needed for formation of a visible specific precipitate.

As shown by Heidelberger *et al.* (33-37), the fixation of complement by antigen-antibody complexes also can be studied by the quantitative precipitin method in terms of uptake of complement protein by the specific aggregate. Measurements of this kind have been made with the blood serum from guinea pigs (33, 34), human beings (37), cattle (38) and rabbits (39, 40). Fixation of complement has been investigated also with radioisotope-labelled complement (41, 42), by the fluorescent staining technic (43), and by means of antisera to complement (44).

In view of the multi-component nature of complement, the significance of these studies is not clear at present. It is believed that C'1 and C'4 combine with antigen-antibody complexes (36, 44) and thus contribute mass to the immune aggregate. C'2 is "fixed" in the sense that its activity disappears in complement fixation, but it is not known whether this component enters into a physical union with immune aggregates. The fixation of C'3a and C'3b is also not settled; these two factors were discovered only recently and adequate fixation studies have not yet been made. From studies involving C'3 as a whole, there is evidence that a physical union is formed (45). Soluble antigen-antibody complexes are also capable of fixing complement, provided they contain more than one molecule of antibody and more than two molecules of antigen (46). Furthermore, aggregated human gamma globulin fixes complement (47, 48). A more extensive treatment of these problems will be given after the components of complement have been discussed in detail.

The ease with which the hemolytic reaction can be observed and measured accurately has led to its widespread use for the detection and assay of complement. Much of the information now available on the nature and mode of action of complement has come from studies of the hemolytic reaction, and most of these basic investigations have been made with a

model system, the use of fresh source of technical of this refined complement the process

In the activity has of the same will produce portion in recent recognition hemolysis region of that the complement cells lyse

(33-37), antigen-studied method in protein by means of the blood of human (39, 40). investigated complement stain-antisera

the nature of these is believed antigenous congregate activity, but it is a component immune and C'3b is were adequate made. whole, union antibody is greater than further-lobulin extent will be element

the reaction used for element. available of complement basic with a

model system comprising sheep erythrocytes, the corresponding rabbit antibody and fresh guinea pig serum as a potent source of complement. At present, the technical procedures developed in the study of this model system represent the most refined methods available for the study of complement, and, accordingly, these are the procedures which will be described in

detail. However, the essential concepts and experimental approaches which have been developed with the aid of the hemolytic model system, as set forth in the present chapter, are amenable to general application and may serve as a guide to the study of the various immunological phenomena involving complement.

### HEMOLYTIC ASSAY OF COMPLEMENT

In the past, hemolytic complement activity has been estimated usually in terms of the smallest amount of fresh serum which will produce complete lysis of a specified portion of sensitized red cells. However, in recent years there has been increased recognition of the advantage of 50% hemolysis as the endpoint. Studies of the region of partial hemolysis have shown that the relation between the amount of complement used and the proportion of cells lysed is not linear, but follows a sig-

moidal curve, as shown in Figure 35. It is evident from the shape of this curve that complete (100%) hemolysis is approached only gradually, and thus, relatively large increases in complement are required to effect lysis of the last 5 to 10% of the cells. In the central region, however, the curve is steep, and the degree of lysis is sensitive to small changes in the amount of complement. For precise titration of the hemolytic activity of complement, the endpoint is therefore chosen in the central part of the

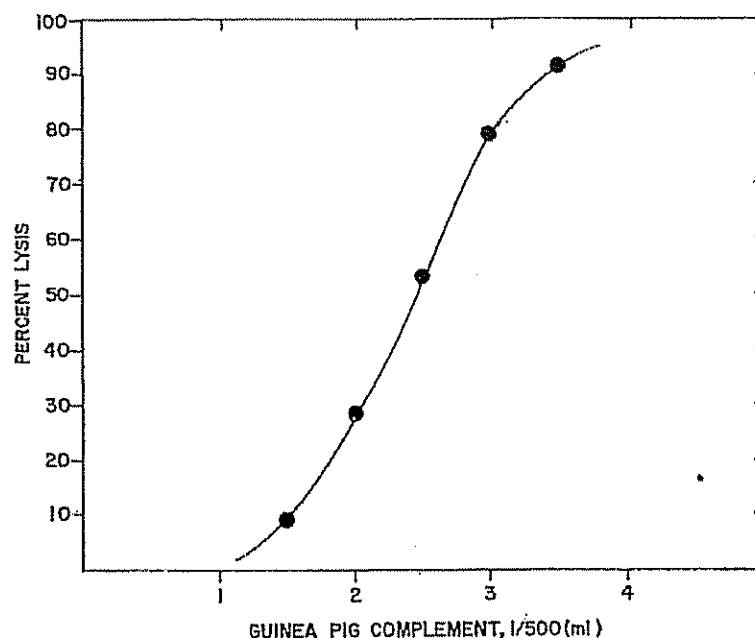


FIG. 35. Percentage of hemolysis of optimally sensitized sheep erythrocytes plotted as a function of the volume of diluted guinea pig complement. One hour incubation at 37°C.

sigmoidal response curve, usually at 50%. Wadsworth, Maltaner and collaborators were among the first to put this to practical use in their quantitative method of complement fixation (49-53).

The 50% hemolytic unit of complement, designated  $C'H_{50}$ , is defined as the quantity of complement required for 50% lysis. This is an arbitrary unit, since its magnitude depends on the concentration of red cells, the fragility of the cells, the quantity of antibody used for sensitization, the nature of that antibody, the ionic strength of the reaction system, the concentrations of  $Ca^{++}$  and  $Mg^{++}$ , pH, reaction time and temperature. These factors must be controlled carefully in hemolytic complement titrations, as discussed in detail below.

For mathematical description of the sigmoidal response curve of the hemolytic reaction, the equation of von Krogh (54)

$$x = K \left( \frac{y}{1-y} \right)^{1/n} \quad [1]$$

has been commonly employed. In this relation,  $x$  represents the amount of complement (expressed in ml. of diluted guinea pig serum) and  $y$  stands for the degree of lysis (i.e., 100  $y$  = per cent hemolysis).

The constant  $K$  is the 50% unit of complement, since at this point  $y = 0.5$ , and the term  $\frac{y}{1-y}$  = unity, and therefore  $x = K$ .

The magnitude of the exponent,  $1/n$ , which determines the shape of the sigmoidal curve, depends on experimental conditions, but, usually, a value of  $0.2 \pm 10\%$  is applicable. One of the factors influencing  $1/n$  is the concentration of sensitized erythrocytes; as shown in Table 1,  $1/n$

increases as the cell concentration is raised; simultaneously, the hemolytic efficiency of the complement goes up, as seen from the decline in the value of  $K$ /cell. It has also been noted that  $1/n$  increases if the concentration of  $Ca^{++}$  is raised.

Furthermore, it has been found that  $1/n$  deviates from 0.2 when the different amounts of complement distributed into tubes for titration are subjected to incubation at  $37^\circ C$ . prior to addition of sensitized red cells. This can be ascribed to the fact that the loss of complement activity which occurs during incubation is more marked in higher dilutions, so that the different amounts of complement set up for titration do not deteriorate uniformly. As a consequence, the quantities of active complement remaining after incubation are no longer related to one another in the proportions in which they were distributed in the tubes, resulting in a value of  $1/n$  differing from 0.2.

Logarithmic transformation of the von Krogh equation furnishes a function which is convenient for evaluation of experimental results:

$$\log x = \log K + \frac{1}{n} \log \left( \frac{y}{1-y} \right) \quad [2]$$

If  $\log x$  is plotted against  $\log \left( \frac{y}{1-y} \right)$ , equation [2] describes a straight line of intercept  $\log K$  and slope  $1/n$ , as shown in Figure 36. In hemolytic assays of complement activity the amounts of complement should be chosen so that the titration yields at least two points in the partial range of lysis, preferably between 20% and 80%. By plotting experimental results according to [2], one obtains  $K$ , which is the quantity

TABLE 1  
Variation of  $1/n$  and  $C'$  Unit ( $K$ ) with Red Cell Concentration

| Red cell concentration, billions/ml.             | 0.06 | 0.12 | 0.25 | 0.5  | 1.0  | 2.0  |
|--------------------------------------------------|------|------|------|------|------|------|
| $1/n$                                            | 0.19 | 0.23 | 0.27 | 0.31 | 0.38 | 0.43 |
| $\frac{K}{\text{cell}} \times 10^3, \text{ ml.}$ | 52.7 | 36.7 | 26.3 | 18.5 | 14.0 | 10.6 |

## Complement and Complement Fixation

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TABLE 2  
y/1-y VALUES

|    | 0     | .1    | .2    | .3    | .4    | .5    | .6    | .7    | .8    | .9    |    |
|----|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|----|
| 10 | .111  | .112  | .114  | .115  | .116  | .117  | .119  | .120  | .121  | .122  | 10 |
| 11 | .124  | .125  | .126  | .127  | .129  | .130  | .131  | .133  | .134  | .135  | 11 |
| 12 | .136  | .138  | .139  | .140  | .142  | .143  | .144  | .145  | .147  | .148  | 12 |
| 13 | .149  | .151  | .152  | .153  | .155  | .156  | .157  | .159  | .160  | .161  | 13 |
| 14 | .163  | .164  | .166  | .167  | .168  | .170  | .171  | .172  | .174  | .175  | 14 |
| 15 | .176  | .178  | .179  | .181  | .182  | .183  | .185  | .186  | .188  | .189  | 15 |
| 16 | .190  | .192  | .193  | .195  | .196  | .198  | .199  | .200  | .202  | .203  | 16 |
| 17 | .205  | .206  | .208  | .209  | .211  | .212  | .214  | .215  | .217  | .218  | 17 |
| 18 | .220  | .221  | .222  | .224  | .225  | .227  | .229  | .230  | .232  | .233  | 18 |
| 19 | .235  | .236  | .238  | .239  | .241  | .242  | .244  | .245  | .247  | .248  | 19 |
| 20 | .250  | .252  | .253  | .255  | .256  | .258  | .259  | .261  | .263  | .264  | 20 |
| 21 | .266  | .267  | .269  | .271  | .272  | .274  | .276  | .277  | .279  | .280  | 21 |
| 22 | .282  | .284  | .285  | .287  | .289  | .290  | .292  | .294  | .295  | .297  | 22 |
| 23 | .299  | .300  | .302  | .304  | .305  | .307  | .309  | .311  | .312  | .314  | 23 |
| 24 | .316  | .318  | .319  | .321  | .323  | .325  | .326  | .328  | .330  | .332  | 24 |
| 25 | .333  | .335  | .337  | .339  | .340  | .342  | .344  | .346  | .348  | .350  | 25 |
| 26 | .351  | .353  | .355  | .357  | .359  | .361  | .362  | .364  | .366  | .368  | 26 |
| 27 | .370  | .372  | .374  | .376  | .377  | .379  | .381  | .383  | .385  | .387  | 27 |
| 28 | .389  | .391  | .393  | .395  | .397  | .399  | .401  | .403  | .404  | .406  | 28 |
| 29 | .408  | .410  | .412  | .414  | .416  | .418  | .420  | .422  | .425  | .427  | 29 |
| 30 | .429  | .431  | .433  | .435  | .437  | .439  | .441  | .443  | .445  | .447  | 30 |
| 31 | .449  | .451  | .453  | .456  | .458  | .460  | .462  | .464  | .466  | .468  | 31 |
| 32 | .471  | .473  | .475  | .477  | .479  | .481  | .484  | .486  | .488  | .490  | 32 |
| 33 | .493  | .495  | .497  | .499  | .502  | .504  | .506  | .508  | .511  | .513  | 33 |
| 34 | .515  | .517  | .520  | .522  | .524  | .527  | .529  | .531  | .534  | .536  | 34 |
| 35 | .538  | .541  | .543  | .546  | .548  | .550  | .553  | .555  | .558  | .560  | 35 |
| 36 | .563  | .565  | .567  | .570  | .572  | .575  | .577  | .580  | .582  | .585  | 36 |
| 37 | .587  | .590  | .592  | .595  | .597  | .600  | .603  | .605  | .608  | .610  | 37 |
| 38 | .613  | .616  | .618  | .621  | .623  | .626  | .629  | .631  | .634  | .637  | 38 |
| 39 | .639  | .642  | .645  | .647  | .650  | .653  | .656  | .658  | .661  | .664  | 39 |
| 40 | .667  | .669  | .672  | .675  | .678  | .681  | .684  | .686  | .689  | .692  | 40 |
| 41 | .695  | .698  | .701  | .704  | .706  | .709  | .712  | .715  | .718  | .721  | 41 |
| 42 | .724  | .727  | .730  | .733  | .736  | .739  | .742  | .745  | .748  | .751  | 42 |
| 43 | .754  | .757  | .761  | .766  | .767  | .770  | .773  | .776  | .779  | .783  | 43 |
| 44 | .786  | .789  | .792  | .795  | .799  | .802  | .805  | .808  | .812  | .815  | 44 |
| 45 | .818  | .822  | .825  | .828  | .832  | .835  | .838  | .842  | .845  | .848  | 45 |
| 46 | .852  | .855  | .859  | .862  | .866  | .869  | .873  | .876  | .880  | .883  | 46 |
| 47 | .887  | .890  | .894  | .898  | .901  | .905  | .908  | .912  | .916  | .919  | 47 |
| 48 | .923  | .927  | .931  | .934  | .938  | .942  | .946  | .949  | .953  | .957  | 48 |
| 49 | .961  | .965  | .969  | .972  | .976  | .980  | .984  | .988  | .992  | .996  | 49 |
| 50 | 1.000 | 1.004 | 1.008 | 1.012 | 1.016 | 1.020 | 1.024 | 1.028 | 1.033 | 1.037 | 50 |
| 51 | 1.041 | 1.045 | 1.049 | 1.053 | 1.058 | 1.062 | 1.066 | 1.070 | 1.075 | 1.079 | 51 |
| 52 | 1.083 | 1.088 | 1.092 | 1.096 | 1.101 | 1.105 | 1.110 | 1.114 | 1.119 | 1.123 | 52 |
| 53 | 1.128 | 1.132 | 1.137 | 1.141 | 1.146 | 1.151 | 1.155 | 1.160 | 1.165 | 1.169 | 53 |
| 54 | 1.174 | 1.179 | 1.183 | 1.188 | 1.193 | 1.198 | 1.203 | 1.208 | 1.212 | 1.217 | 54 |
| 55 | 1.222 | 1.227 | 1.232 | 1.237 | 1.242 | 1.247 | 1.252 | 1.257 | 1.262 | 1.268 | 55 |
| 56 | 1.273 | 1.278 | 1.283 | 1.288 | 1.294 | 1.299 | 1.304 | 1.309 | 1.315 | 1.320 | 56 |
| 57 | 1.326 | 1.331 | 1.336 | 1.342 | 1.348 | 1.353 | 1.358 | 1.364 | 1.370 | 1.375 | 57 |
| 58 | 1.381 | 1.387 | 1.392 | 1.398 | 1.404 | 1.410 | 1.415 | 1.421 | 1.427 | 1.433 | 58 |
| 59 | 1.439 | 1.445 | 1.451 | 1.457 | 1.463 | 1.469 | 1.475 | 1.481 | 1.488 | 1.494 | 59 |
| 60 | 1.500 | 1.506 | 1.513 | 1.519 | 1.525 | 1.532 | 1.538 | 1.545 | 1.551 | 1.558 | 60 |
| 61 | 1.564 | 1.571 | 1.577 | 1.584 | 1.591 | 1.597 | 1.604 | 1.611 | 1.618 | 1.625 | 61 |
| 62 | 1.632 | 1.639 | 1.646 | 1.653 | 1.660 | 1.667 | 1.674 | 1.681 | 1.688 | 1.695 | 62 |
| 63 | 1.703 | 1.710 | 1.717 | 1.725 | 1.732 | 1.740 | 1.747 | 1.755 | 1.762 | 1.770 | 63 |
| 64 | 1.778 | 1.786 | 1.793 | 1.801 | 1.809 | 1.817 | 1.825 | 1.833 | 1.841 | 1.849 | 64 |

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TABLE 2—Cont'd

|    | 0     | .1    | .2    | .3    | .4    | .5    | .6    | .7    | .8    | .9    |    |
|----|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|----|
| 65 | 1.857 | 1.865 | 1.874 | 1.882 | 1.890 | 1.899 | 1.907 | 1.915 | 1.924 | 1.933 | 65 |
| 66 | 1.941 | 1.950 | 1.959 | 1.967 | 1.976 | 1.985 | 1.994 | 2.003 | 2.012 | 2.021 | 66 |
| 67 | 2.030 | 2.040 | 2.049 | 2.058 | 2.067 | 2.077 | 2.086 | 2.096 | 2.106 | 2.115 | 67 |
| 68 | 2.125 | 2.135 | 2.145 | 2.155 | 2.165 | 2.175 | 2.185 | 2.195 | 2.205 | 2.215 | 68 |
| 69 | 2.226 | 2.240 | 2.247 | 2.257 | 2.268 | 2.279 | 2.289 | 2.300 | 2.311 | 2.322 | 69 |
| 70 | 2.333 | 2.344 | 2.356 | 2.367 | 2.378 | 2.390 | 2.401 | 2.413 | 2.425 | 2.436 | 70 |
| 71 | 2.448 | 2.460 | 2.472 | 2.484 | 2.497 | 2.509 | 2.521 | 2.534 | 2.546 | 2.559 | 71 |
| 72 | 2.571 | 2.584 | 2.597 | 2.610 | 2.623 | 2.636 | 2.650 | 2.663 | 2.676 | 2.690 | 72 |
| 73 | 2.704 | 2.717 | 2.731 | 2.745 | 2.759 | 2.774 | 2.788 | 2.802 | 2.817 | 2.831 | 73 |
| 74 | 2.846 | 2.861 | 2.876 | 2.891 | 2.906 | 2.922 | 2.937 | 2.953 | 2.968 | 2.984 | 74 |
| 75 | 3.000 | 3.016 | 3.032 | 3.049 | 3.065 | 3.082 | 3.098 | 3.115 | 3.132 | 3.149 | 75 |
| 76 | 3.167 | 3.184 | 3.202 | 3.219 | 3.237 | 3.255 | 3.274 | 3.292 | 3.310 | 3.329 | 76 |
| 77 | 3.348 | 3.367 | 3.386 | 3.405 | 3.425 | 3.444 | 3.464 | 3.484 | 3.505 | 3.525 | 77 |
| 78 | 3.545 | 3.566 | 3.587 | 3.608 | 3.630 | 3.651 | 3.673 | 3.695 | 3.717 | 3.739 | 78 |
| 79 | 3.762 | 3.785 | 3.808 | 3.831 | 3.854 | 3.878 | 3.902 | 3.926 | 3.950 | 3.975 | 79 |
| 80 | 4.000 | 4.025 | 4.050 | 4.076 | 4.102 | 4.128 | 4.155 | 4.181 | 4.208 | 4.236 | 80 |
| 81 | 4.263 | 4.291 | 4.319 | 4.348 | 4.376 | 4.405 | 4.435 | 4.464 | 4.495 | 4.525 | 81 |
| 82 | 4.556 | 4.587 | 4.618 | 4.650 | 4.682 | 4.714 | 4.747 | 4.780 | 4.814 | 4.848 | 82 |
| 83 | 4.882 | 4.917 | 4.952 | 4.988 | 5.024 | 5.061 | 5.098 | 5.135 | 5.173 | 5.211 | 83 |
| 84 | 5.250 | 5.289 | 5.329 | 5.369 | 5.410 | 5.452 | 5.494 | 5.536 | 5.579 | 5.623 | 84 |
| 85 | 5.667 | 5.711 | 5.757 | 5.803 | 5.849 | 5.897 | 5.944 | 5.993 | 6.042 | 6.092 | 85 |
| 86 | 6.143 | 6.194 | 6.246 | 6.299 | 6.353 | 6.407 | 6.463 | 6.519 | 6.576 | 6.634 | 86 |
| 87 | 6.692 | 6.752 | 6.813 | 6.874 | 6.937 | 7.000 | 7.065 | 7.130 | 7.197 | 7.264 | 87 |
| 88 | 7.333 | 7.403 | 7.475 | 7.547 | 7.621 | 7.696 | 7.772 | 7.850 | 7.929 | 8.009 | 88 |
| 89 | 8.091 | 8.174 | 8.259 | 8.346 | 8.434 | 8.524 | 8.615 | 8.709 | 8.804 | 8.901 | 89 |
| 90 | 9.000 | 9.101 | 9.204 | 9.310 | 9.417 | 9.526 | 9.638 | 9.753 | 9.870 | 9.989 | 90 |

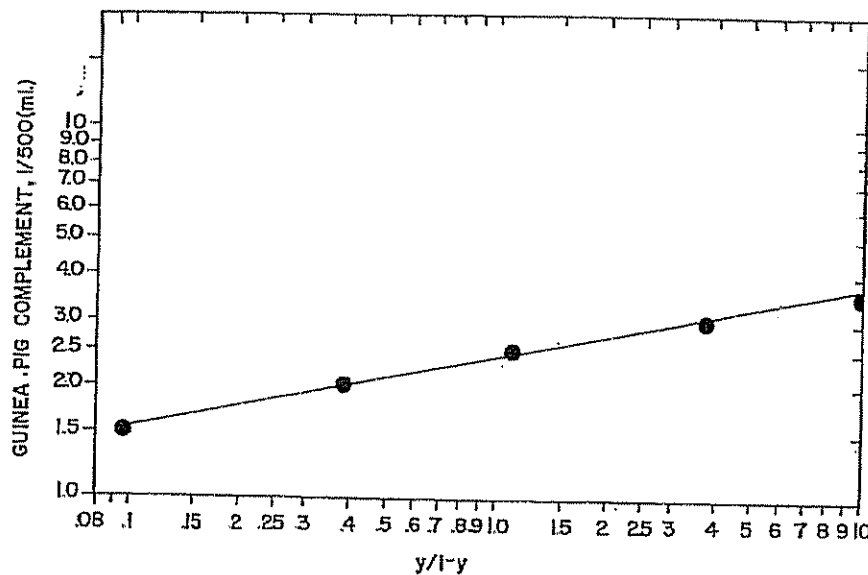


FIG. 36. Hemolysis of optimally sensitized sheep erythrocytes by guinea pig complement. Logarithmic plot of  $x$  against  $y/1-y$ , where  $x$  = ml of diluted complement and  $y$  = degree of lysis. Ordinate =  $x$ . Abscissa =  $y/1-y$ .

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of complement required for 50% lysis, i.e.,  $C'H_{50}$ , as well as  $1/n$ , which furnishes a check on the validity of the titration. For convenience in the evaluation of experimental results by equation [2] values for  $\frac{y}{1-y}$  are given in Table 2.

Sometimes, only a single experimental point in the region of partial lysis is available, and in this event, the 50% unit can be calculated, provided the value of  $1/n$  is known. If the titrations are conducted under conditions where  $1/n = 0.2$ , such single point analyses may be evaluated with the aid of the conversion factors given in Table 3. These factors are obtained as follows: If the amount of complement required for 50% lysis is assigned the value 1, and if  $1/n = 0.2$  is substituted in equation [1] the expression

$$x = \left( \frac{y}{1-y} \right)^{0.2} \quad [3]$$

is obtained, which relates  $x$ , the amount of complement giving any degree of lysis ( $y$ ) to 1, the quantity of complement required

TABLE 3

Conversion Factors Calculated from the Von Krogh Equation for  $1/n = 0.2$

| Degr. of lysis | Factor | Degree of lysis | Factor |
|----------------|--------|-----------------|--------|
| 0.10           | 0.644  | 0.55            | 1.041  |
| 0.12           | 0.671  | 0.60            | 1.084  |
| 0.14           | 0.696  | 0.65            | 1.132  |
| 0.16           | 0.718  | 0.70            | 1.185  |
| 0.18           | 0.738  | 0.75            | 1.246  |
| 0.20           | 0.758  | 0.80            | 1.320  |
| 0.25           | 0.803  | 0.82            | 1.354  |
| 0.30           | 0.844  | 0.84            | 1.393  |
| 0.35           | 0.884  | 0.86            | 1.438  |
| 0.40           | 0.922  | 0.88            | 1.490  |
| 0.45           | 0.961  | 0.90            | 1.552  |
| 0.50           | 1.000  |                 |        |

for 50% lysis. The conversion factors given in Table 3, represent the values of  $x$  for the range  $y = 0.10$  to  $y = 0.90$ .

The use of these conversion factors may be illustrated as follows: For example, if 1.0 ml. of a  $1/250$  dilution of complement produces 30% lysis (i.e.,  $y = 0.30$ ), the number of  $C'H_{50}$  per ml. of undiluted complement equals  $250 \times 0.844 = 211$  units.

#### KINETICS OF HEMOLYSIS BY ANTIBODY AND COMPLEMENT

During recent years a better understanding of the mechanism of the hemolytic action of complement has been achieved through the study of its kinetics. This was attempted by some of the early students of complement, but their efforts were handicapped by lack of suitable apparatus and technic. With the development of photometric methods for the measurement of hemolysis (55), as well as the recognition of the role of  $Ca^{++}$  and  $Mg^{++}$  (56), it became possible to devise suitable technical procedures (57).

In a typical experiment, a 125 ml. Erlenmeyer flask is charged with a suspension of washed sheep erythrocytes in isotonic veronal-NaCl buffer containing 0.00015  $M$   $Ca^{++}$  and 0.0005  $M$   $Mg^{++}$ , which are optimal concentrations of these divalent cations for hemolysis by rabbit antibody

and guinea pig complement. The buffer also contains 0.1% crystallized bovine serum albumin or gelatin as stabilizing agents. The flask is suspended from a rocking mechanism in a constant temperature water bath. After temperature equilibration, suitable dilutions of antibody and complement are added in the sequence and manner required by the purpose of the experiment (usually complement is added at zero-time to the sensitized cells). The reaction mixture is shaken continuously to maintain the cells in uniform suspension during the experiment. At suitable intervals of time, samples are withdrawn by means of a pipette and are transferred to a test tube containing ice-cold citrate-saline, or preferably,  $Na_2H$ -EDTA-saline (ethylene-diamine tetraacetate), followed by mixing. The samples are

centrifuged immediately, and the degree of lysis in each sample is determined by photometric analysis of the supernatant fluid for oxyhemoglobin.

The crucial element in kinetic analyses of this type is the method of sampling; the sampling procedure should be designed to stop the hemolytic action abruptly, and to keep it arrested until the cells have been separated from the fluid phase. In a reaction with optimally sensitized cells (see next section) and complement as the limiting factor, the so-called "limited complement system;" dilution and chilling are the only practical methods of stopping; the use of chelating agents, such as EDTA, for binding of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ , is ineffective, because in the "limited complement system" those reaction steps which require  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  take place during the first few minutes of the reaction, and thereafter  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  no longer play a role. (This is discussed in greater detail in a later section.) However, the stopping efficacy of dilution and chilling is not en-

tirely adequate, and therefore samples must be centrifuged immediately after withdrawal from the reaction mixture and they must be kept cold during centrifugation.

The "limited antibody system" refers to a reaction in which relatively little antibody is used, and the concentration of complement is increased commensurately. Under these conditions, chelating agents are effective in arresting the reaction process, and consequently the performance of kinetic analyses is technically simpler.

In experiments of long duration, the slow conversion of oxyhemoglobin to methemoglobin, which has a different light absorption spectrum, leads to analytical error unless adequate corrections are made. Oxyhemoglobin is characterized by light absorption maxima at 576, 541 and 414  $\text{m}\mu$ . In most quantitative studies in recent years the peak at 541  $\text{m}\mu$  has been used, but in experiments of many hours' duration the error due to methemoglobin formation becomes appreciable (about 4% in 8 hours

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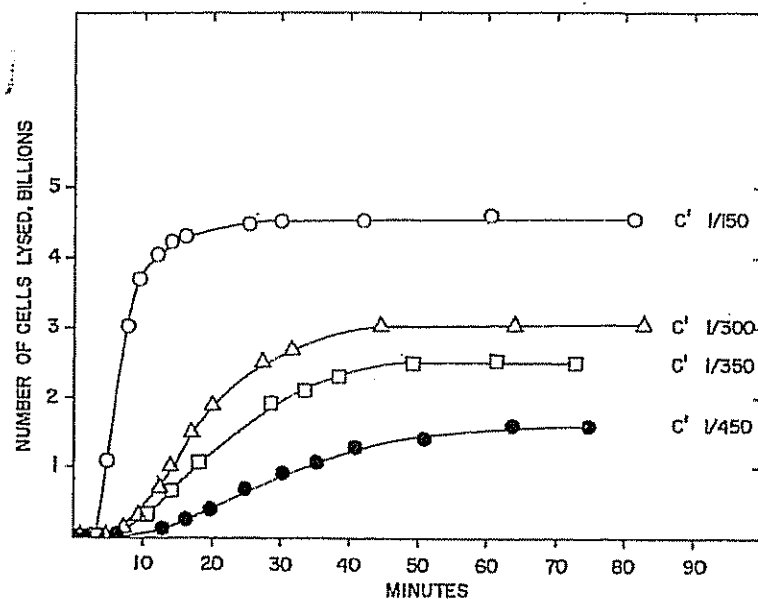


FIG. 37. Kinetics of hemolysis of optimally sensitized sheep erythrocytes by guinea pig complement (optimal  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ ; 37°C.). From (57).

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at 37° C.). Therefore, in prolonged experi-  
ments it is preferable to make readings  
at 412 m $\mu$ , where the absorption spectra  
of oxy- and methemoglobin intersect.

A typical series of kinetic hemolysis  
curves, showing the reaction of "optimally"  
sensitized sheep erythrocytes with guinea  
pig complement at 37°C, is illustrated in  
Figure 37 (limited complement system).  
The curves are sigmoidal, i.e., the speed  
of lysis starts from zero, increases to a  
maximal value which depends on the con-  
centration of complement, and then de-  
clines again to zero. At 37°C. the lytic  
reaction is finished in about 90 minutes,  
and this is the usual incubation-period for  
endpoint titrations of guinea pig comple-  
ment.

As shown in Figure 38, the kinetic hemo-  
lysis curve for human complement is also  
sigmoidal, but it differs from the guinea pig  
curves in respect to the fact that an end-  
point is not reached even after several  
hours of incubation at 37°C. Therefore, in  
titrations of human complement, the time  
of incubation represents an arbitrary fac-  
tor.

Similarly, in the "limited antibody"  
system an endpoint is not reached, even

after many hours of incubation, as shown  
in Figures 39 and 40. This must be taken  
into account in the titration of hemolytic  
antibody. The problem is complicated  
further by the fact that kinetic hemolysis  
curves obtained with different antisera may  
be dissimilar in shape, as illustrated in  
Figures 39 and 40. This effect can be vis-  
ualized more readily if antisera of dissimilar  
behavior are studied at dilutions chosen so  
as to yield superimposable kinetic curves  
during the early stages of reaction. Such an  
experiment is illustrated in Figure 41, with  
four antisera, one (G) prepared by inocu-  
lation of rabbits with sheep erythrocyte  
stromata, the other three made with  
washed intact red cells as antigen. If two  
antisera like A and G are compared in a  
titration with incubation for thirty min-  
utes at 37°C., G will yield the higher titer;  
on the other hand, in a two hour titration  
A will appear to be more potent.

From studies by Bowman, Mayer and  
Rapp (58), as well as earlier observations  
(16, pages 34 to 38), such differences in  
kinetic behavior probably can be attrib-  
uted to dissociation of antibody molecules  
from the erythrocyte-antibody complex,  
followed by reassociation with other anti-

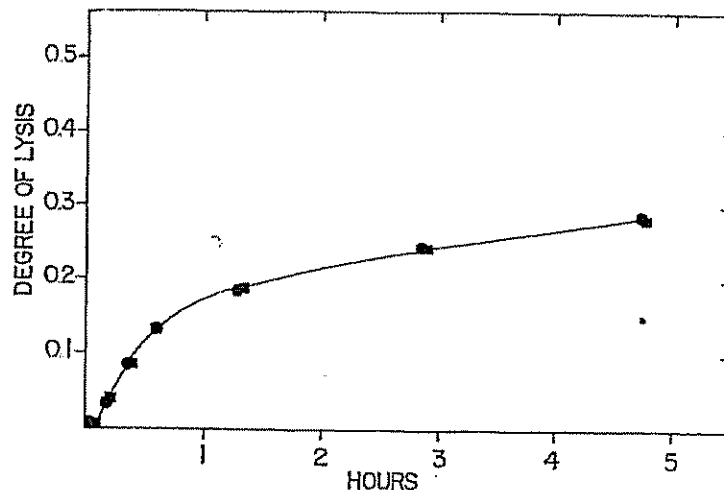


FIG. 38. Kinetics of hemolysis of optimally sensitized sheep erythrocytes by human complement at 37°C.

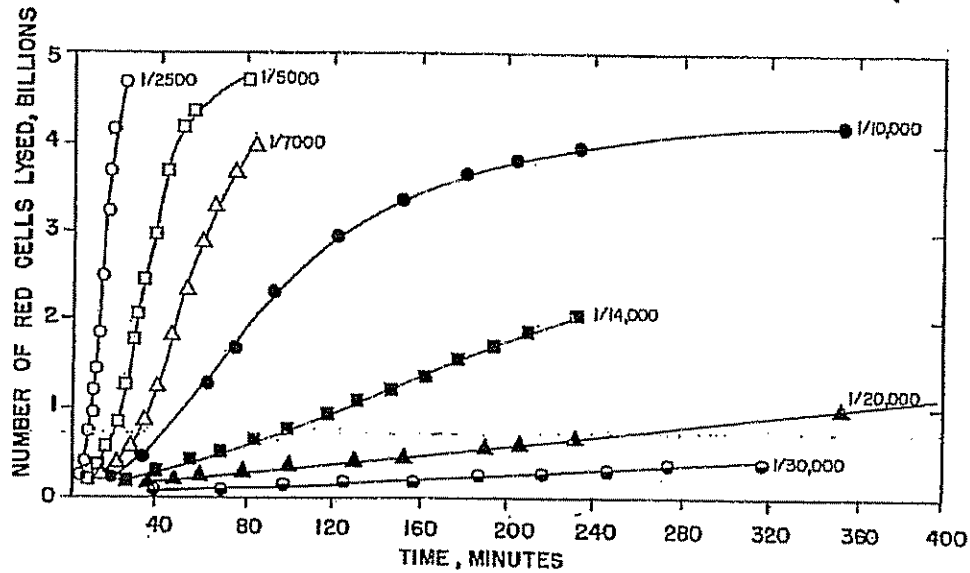


FIG. 39. Kinetic hemolysis curves with constant excess of guinea pig complement and varying dilutions of rabbit antiserum to sheep erythrocytes (limited antibody; optimal  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ ;  $37^\circ\text{C}$ .) (from ref. 57).

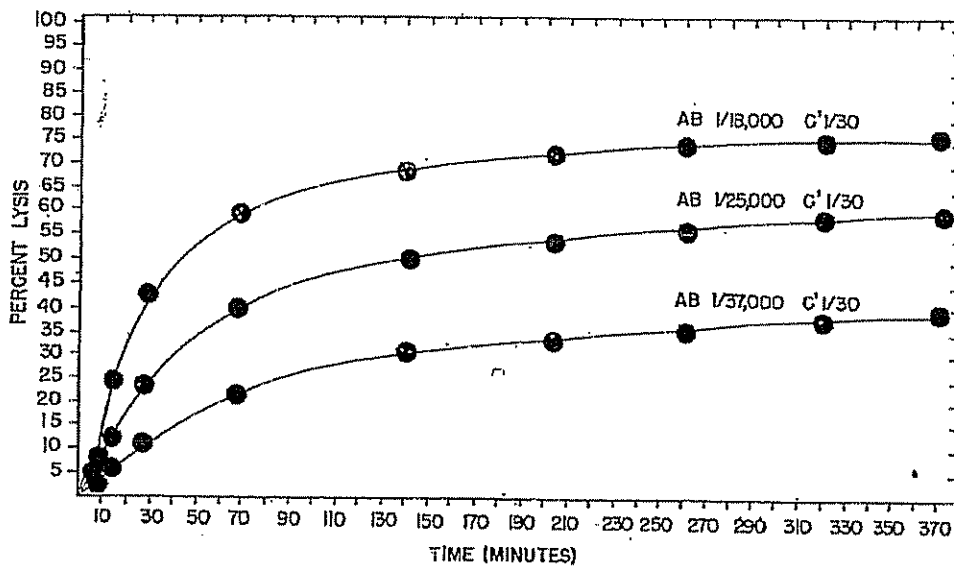


FIG. 40. Kinetic hemolysis curves with constant excess of guinea pig complement and varying dilutions of rabbit antiserum to boiled sheep erythrocyte stromata (limited antibody; optimal  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ ;  $37^\circ\text{C}$ .) From (23).

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## Complement and Complement Fixation

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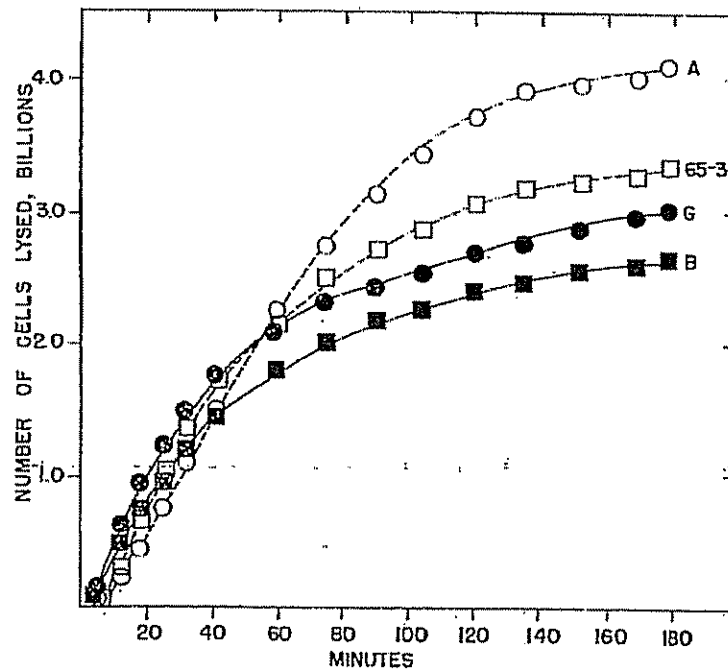


FIG. 41. Kinetic hemolysis curves with excess complement and limited antibody, showing the dissimilarity in kinetic behavior of different antisera. The antibody in "A" and "65-3" showed marked dissociation tendency, while that in "G" and "B" did not (from ref. 58).

gen sites, either on the same, or on another cell. This gives rise to continuous transfer of antibody from site to site on the same cell, or from cell to cell, during the hemolytic reaction, a kind of cycling effect enabling a given antibody molecule to act repeatedly. Some antisera display this effect, while others do not. In the absence of antibody transfer or cycling at an appreciable rate, any red cells which did not pick up enough antibody during the sensitization cannot react with complement and therefore do not hemolyze. On the other hand, with an antiserum showing appreciable transfer tendency, even cells which had inadequate antibody at the beginning of the experiment will eventually pick up enough, and as a result, will react with the components of the complement system.

A direct demonstration of the transfer effect can be seen in a kinetic analysis of

the type shown in Figure 42. In essence, the experiment involves the use of a cell suspension sensitized with an appropriate antiserum at a dilution chosen so that there is no excess of antibody, i.e., the fluid phase is free of demonstrable antibody. Excess complement is added and the reaction mixture is incubated at 37°C. until most or all of the cells are lysed. A new supply of cells, not sensitized with antibody, is then added (see arrow), and on further incubation these cells will be hemolyzed, provided the antibody used for sensitization of the first portion of cells has an appreciable transfer tendency.

In kinetic studies of the limited antibody system with Cr<sup>51</sup> labelled erythrocytes by Weinrach *et al.* (59), the maximal rate of hemolysis was found to vary with the square of the antibody concentration; from this it was concluded that two mole-

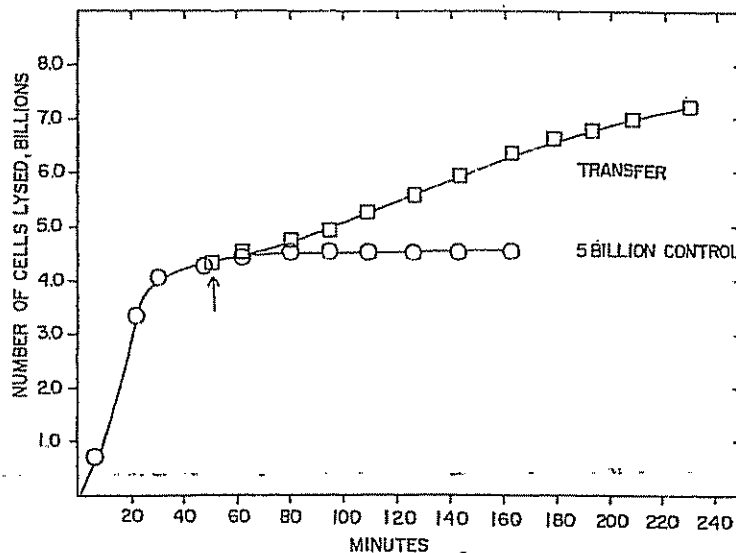


FIG. 42. Kinetic experiment showing hemolysis of a portion of unsensitized cells which were added to the reaction system when the initial population of sensitized cells had lysed about 90% (from ref. 58).

cules of hemolytic antibody of  $S = 18$  variety ( $\gamma_1$ ), in close proximity, are required for a sensitized site at the cell surface. Moreover, it was postulated that bimolecular complexes are more stable on the red cells than a single antibody molecule. As a consequence, there would be a tendency toward formation of bimolecular complexes through migration of single antibody molecules from site to site, until, by chance, a more stable bimolecular configuration is formed. If cells, excess complement, and limited antibody are mixed in that order, there is a long lag period which is attributed to the formation of bimolecular complexes (60).

This concept is of interest in connection with Heidelberger's suggestion (33) that complement forms loose complexes with antibody and that these are stabilized when an aggregate is formed through reaction with antigen. In accord with this hypothesis, Drs. T. and K. Ishizaka (47) have found that heat-denatured, aggregated human gamma globulin fixes complement.

It has been shown that the hemolytic efficiency of hemolytic antisera may vary widely relative to their agglutinating titer, cell receptor site blocking capacity (61), or antibody weight content (62-65). This has been interpreted as due to the presence, in varying proportion of at least two antibodies which differ in hemolytic efficiency (63). Stelos *et al.* (65) and Talmage *et al.* (66) separated  $\gamma_1$  globulin, representing hemolytically efficient large molecules, from the  $\gamma_2$  globulin fraction containing mostly small, hemolytically inefficient antibody molecules. The rate of hemolysis with the  $\gamma_2$  fraction was found to vary with the fourth power of its concentration, suggesting that for this antibody a tetramolecular complex is necessary for sensitization of a site on the cell surface, in contrast to the postulated bimolecular complex for the  $\gamma_1$  antibody. The validity of these concepts has not been proven rigorously, but, if correct, they would explain the lower hemolytic efficiency of the  $\gamma_2$  antibody.

It is postulated by Weinrach and Tal-

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mage (60) that on sensitization of cells with antibody, prior to addition of complement, bimolecular (or tetramolecular) complexes accumulate through site-to-site transfer. When complement is added, these multimolecular antibody sites react rapidly until exhausted. Thereafter, reaction with complement proceeds at a slower rate, paced by the speed of complex formation through transfer. If cells are mixed with complement, followed by antibody, there is a long lag during which multimolecular complexes are believed to accumulate. In either case, the speed of antibody transfer would play an important role.

Bowman *et al.* (58) found that some antisera (anti-stromata sera) did not show transfer from cell to cell at a measurable rate, and Rapp (67) showed that as few as 10 molecules of such antibody suffice for lysis of a single cell in the presence of excess complement. This may be in conflict with Weinrach and Talmage's hypothesis, but it is possible that the rate of site-to-site transfer on the same cell may be appreciably higher than the cell-to-cell rate, and if so, the probability of formation of bimolecular complexes would be increased. It has been observed by Borsos *et al.* (68) that the number of cell surface sites reactive with complement is considerably less than the number of antibody molecules used for sensitization. This could mean that only a small proportion of the antibody molecules is capable of fixing complement, but alternatively, and perhaps more likely, this can be explained by the hypothesis of Weinrach and Talmage. Precise determinations of the relation between the number of sensitized sites and

the concentration of antibody would furnish rigorous evidence for or against the bimolecular complex hypothesis. This important problem will be discussed further in a later section.

Since hemolysis in the limited antibody system, under the usual experimental conditions, does not reach an endpoint, i.e., does not come to a standstill at any time, a kinetic method of analysis for hemolytic antibody was developed by Croft (62), based on determination of the maximal lytic velocity, i.e., slope of the sigmoidal hemolysis curve at the inflection point (69). For reasons of convenience of measurement, the unit of hemolytic antibody was defined as that amount which produces a maximal lytic velocity of  $3 \times 10^8$  cells per minute, under specific experimental conditions with respect to cell and complement concentration, temperature of incubation,  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  concentration and ionic strength.

Measurements of this nature are quite laborious, and therefore, an abbreviated method has been evolved in which the unit of hemolytic antibody is defined as that amount which produces 50 per cent hemolysis in exactly fifteen minutes following addition of complement. This method of measurement yields titers approximately equal to those based on maximal velocity, with lysis of 300 million cells a minute as reference point. A similar method has been used by W. H. and L. G. Taliaferro (70).

Other aspects of the kinetics of immune hemolysis will be taken up after discussion of the components of complement and their sequential action.

#### FACTORS WHICH INFLUENCE THE HEMOLYTIC ACTION OF COMPLEMENT

##### Effect of Hemolysin Concentration

The hemolytic efficiency of complement depends upon the amount of hemolytic antibody used for sensitization of the cells. In general, hemolytic complement activity

increases with the degree of sensitization, but the reaction patterns of different antisera vary considerably. There are three types, two of which are shown in Figure 43. One of these is a pattern in which a

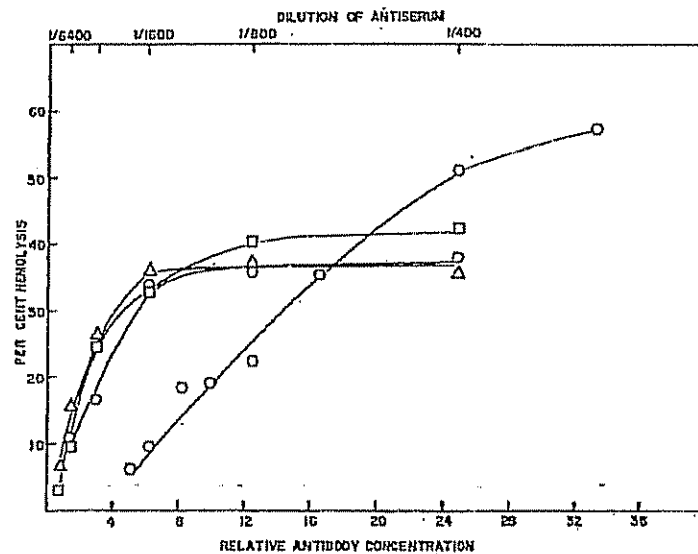


FIG. 43. Per cent hemolysis obtained with a constant amount of C' and increasing concentrations of hemolytic rabbit antisera.

Sera 219 (O), 220 (□) and 221 (Δ) obtained from individual rabbits immunized with boiled sheep red cell stromata. Amboceptor J (◊) constitutes a pool of sera drawn from rabbits immunized with intact, washed sheep erythrocytes (from ref. 76).

constant level of hemolytic complement activity is approached with relatively large amounts of hemolysin (anti-Forssman sera numbers 219, 220 and 221, made by inoculation of rabbits with boiled sheep erythrocyte stromata). The second kind of pattern is exemplified by Pool J in Figure 43. Some antisera yield still another pattern, not shown in Figure 43, in which complement activity rises to a maximum and then declines.

For titration of complement it is convenient to use antisera of the first type. The amount of antibody is chosen in the plateau region so as to yield "optimal sensitization" of the cells. For example, with antisera 219, 220 and 221 (Fig. 43) the minimal amount of antibody yielding "optimal sensitization" would be furnished by dilutions of approximately 1/1200, 1/800 and 1/1200, respectively. Rabbit antisera against boiled sheep erythrocyte stromata (anti-Forssman) usually yield

patterns with a broad plateau region. In addition, such antisera generally exhibit a high ratio of hemolytic to agglutinating activity, another convenient feature.

In titrations of hemolytic complement it is important to take cognizance of the natural occurrence of hemolysins in the blood of most animals. Usually these normal hemolysins are present in low titer. In the titration of guinea pig complement this is not a serious problem because of its relatively high complement titer. On the other hand, with human serum, or with other animal sera exhibiting relatively low hemolytic complement activity, the dilutions needed for hemolytic titration of complement may be within the range where natural hemolysin action is manifest. It should be noted in this connection, that the optimal (plateau) level of sensitization of sheep erythrocytes with Forssman antibody, as shown in Figure 43, does not represent the *maximal* extent of sensitiza-

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tion. For example, amboceptor J (Fig. 43) yielded higher hemolytic complement activity. Furthermore, Carbowax 4000, a poly-ethylene glycol, will enhance sensitization beyond that furnished by antibody (71). In the absence of a known method of *maximal* sensitization of red blood cells, the possibility exists that natural sensitizers present in the serum to be titrated for complement activity may augment the degree of sensitization of cells, thus producing an erroneously high complement titer.

This difficulty may be circumvented, at least in part, by absorption of natural hemolysin with a suspension of washed sheep erythrocytes. This must be done strictly at 0°C. for a short period of time (5-10 minutes) in order to minimize simultaneous fixation of complement. However, even if this is done, there is some uncertainty since animal sera may contain sensitizers which cannot be removed by absorption.

This problem also arises in C' fixation tests in which the patient's serum, especially if used in low dilution, may contribute to the sensitization of the cells. In order to avoid the resulting disturbance of the C' standardization, it is necessary to remove hemolysin from the patient's serum by absorption with red cells.

#### Effect of Red Cell Concentration

As shown in Table 4, in the limited complement system, under certain experimental conditions, the extent of lysis of sensitized red cells, on an *absolute* basis, is *independent* of the concentration of sensitized red cells (quantity of antibody per cell remaining constant). This has been found to apply only in the range from about 100 to 500 molecules of antibody (anti-Forsman) per cell. With substantially higher levels of sensitization, the absolute velocity and extent of lysis tend to increase with the cell concentration,

while deviation in the opposite direction occurs below 100 molecules of antibody per cell, a condition which approaches the limited antibody system.

TABLE 4

*The Absolute Number of Cells Lysed by a Limited Amount of Complement is Essentially Independent of the Total Number of Sensitized Cells in the Reaction System*

|                       |       |      |      |      |      |
|-----------------------|-------|------|------|------|------|
| Total cells, billions | 0.625 | 1.25 | 2.50 | 5.00 | 10.0 |
| Cells lysed, billions | 0.49  | 0.54 | 0.53 | 0.55 | 0.50 |

The amount of complement required for 50% lysis, of course, increases with concentration of sensitized cells. For example, doubling of the cells requires an increase of about 25% in the dose of complement for 50% lysis. The reader is referred to Table 1, from which these relationships can be deduced.

These complex matters are discussed more fully in the theoretical section, with respect to the concept that immune hemolysis is a one-hit process.

#### Effect of Reaction Volume

The hemolytic activity of complement varies inversely with the total reaction volume. If the quantities of sensitized cells and of complement are kept constant, but the volume of the reaction system is increased by addition of extra diluent, the degree of lysis diminishes. This effect is shown in the following tabulation in terms of the change in  $C'H_{50}$  as a function of total reaction volume. As explained in a later section, the cause of this phenomenon lies in the action of C'2 and C'3, the effectiveness of which depends on concentration.

|                                                                                                                          |     |     |     |
|--------------------------------------------------------------------------------------------------------------------------|-----|-----|-----|
| Total Reaction Volume, ml.:                                                                                              | 2.0 | 4.0 | 8.0 |
| Complement Titer, $C'H_{50}/ml.$ :                                                                                       | 387 | 286 | 197 |
| (5 x 10 <sup>8</sup> cells; pH 7.4; 37°C; ionic strength 0.147; 0.00015 M Ca <sup>++</sup> , 0.0005 M Mg <sup>++</sup> ) |     |     |     |

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**Effect of Ionic Strength**

The hemolytic activity of complement varies inversely with the ionic strength of the diluent, as shown in the following tabulation:

|                                                                          |       |       |       |       |       |
|--------------------------------------------------------------------------|-------|-------|-------|-------|-------|
| Ionic Strength:                                                          | 0.140 | 0.146 | 0.158 | 0.167 | 0.177 |
| Complement Titer, C'H <sub>10</sub> :                                    | 220   | 201   | 169   | 143   | 136   |
| (pH 7.4; 37°C.; 0.00015 M Ca <sup>++</sup> , 0.0005 M Mg <sup>++</sup> ) |       |       |       |       |       |

Therefore, in hemolytic titrations of complement the ionic strength of the diluent should not be allowed to vary by more than  $\pm \frac{1}{2}\%$ . Recent experiments by Becker and Wirtz (72) indicate that the action of C'3 is sensitive to salt.

**Effect of Temperature**

Hemolytic reactions have usually been performed at 37°C., but as pointed out by Leon (73), higher titers are obtained in the vicinity of 30°C., due to greater stability of EAC'1, 4, 2 at the lower temperature (see below). However, at 30°C. about three hours are needed to reach the endpoint. The effect of temperature on the hemolytic activity of guinea pig complement is shown in the following tabulation:

|                                                                                                               |      |      |      |      |
|---------------------------------------------------------------------------------------------------------------|------|------|------|------|
| Temperature, °C.                                                                                              | 27.5 | 29.9 | 32.8 | 36.9 |
| Complement Titer, C'H <sub>10</sub> :                                                                         | 229  | 228  | 217  | 174  |
| (3 hours' incubation; pH 7.4; ionic strength 0.147; 0.00015 M Ca <sup>++</sup> , 0.0005 M Mg <sup>++</sup> ). |      |      |      |      |

The choice of incubation temperature will depend on the purpose of the experiment. In the past, most investigators have worked at 37°C., and in most instances it will be convenient to continue this practice since sixty to ninety minutes' incubation suffices to reach the endpoint, at least with guinea pig complement. However, at 37°C. careful control of temperature is necessary ( $\pm 0.1$  or  $0.2^\circ\text{C}.$ ).

**Effect of pH**

The hemolytic activity of complement declines with increase of pH, as shown in the following tabulation:

|                                                                                        |      |      |      |      |
|----------------------------------------------------------------------------------------|------|------|------|------|
| pH                                                                                     | 7.15 | 7.62 | 8.06 | 8.52 |
| Complement Titer, C'H <sub>10</sub> :                                                  | 254  | 233  | 218  | 201  |
| (37°C., ionic strength 0.149; 0.00015 M Ca <sup>++</sup> , 0.0005 M Mg <sup>++</sup> ) |      |      |      |      |

The difference between these data and those presented in the first edition of this text probably arises from the higher concentrations of Ca<sup>++</sup> and Mg<sup>++</sup> and the use of veronal buffer in the present experiment.

**Effect of Ca<sup>++</sup> and Mg<sup>++</sup>**

In confirmation and extension of earlier studies (74), it was found in 1945 that Mg<sup>++</sup> exerts a marked enhancing effect on the hemolytic action of complement (56). It was shown that the titer of guinea pig complement can be increased approximately two-fold by addition of Mg<sup>++</sup> to a final concentration of about 0.0005 M. This means that the hemolytic system, as ordinarily constituted, does not contain sufficient Mg<sup>++</sup> for optimal activity. In the presence of optimal Mg<sup>++</sup>, slight further enhancement of the hemolytic activity of guinea pig complement can be produced by addition of traces of Ca<sup>++</sup>. This is not the case with human, monkey or rat complement; indeed, addition of extra Ca<sup>++</sup> to these sera may exert a slight inhibitory effect (cf. 13).

As described in a later section, Ca<sup>++</sup> functions in the reaction with C'1, while Mg<sup>++</sup> is necessary for action of C'2. It has been found that addition of Ca<sup>++</sup> may interfere with the C'2 reaction step, depending on the concentration of Mg<sup>++</sup>. Conversely, Mg<sup>++</sup> may interfere in the Ca<sup>++</sup> step (75). In the titration of hemolytic complement activity, it is necessary to determine the balance of Ca<sup>++</sup> to Mg<sup>++</sup> yielding optimal activity. As pointed out by Osler *et al.* (13), the cation concentrations proper for guinea pig complement are not necessarily optimal for hemolytic measurement with complements from other species.

Anions like citrate, pyrophosphate, or ethylene - diamine tetraacetate (EDTA), which bind Mg<sup>++</sup> and Ca<sup>++</sup>, inhibit the

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hemolytic action of complement. In practical work with guinea pig complement, such as diagnostic complement fixation, as well as in theoretical studies, addition of Mg<sup>++</sup> and Ca<sup>++</sup> to an optimal level of activity is desirable because a substantial stabilization of the hemolytic system is

thus effected, i.e., there is a marked gain in reproducibility due to the fact that traces of Mg<sup>++</sup>, or of substances which bind Mg<sup>++</sup>, sometimes introduced inadvertently, will not cause fluctuation of hemolytic activity. At high concentration (0.005 M), Mg<sup>++</sup> is inhibitory.

### PROCEDURE FOR THE TITRATION OF COMPLEMENT

The hemolytic unit of complement, CH<sub>50</sub>, is defined as that amount in milliliters which will lyse 2.5 x 10<sup>8</sup> optimally sensitized red cells out of a total of 5 x 10<sup>8</sup> cells, in the presence of optimal Ca<sup>++</sup> and Mg<sup>++</sup>, at an ionic strength of 0.147, in one hour's incubation at 37°C. in a total volume of 7.5 ml.

#### Reagents (57, 62, 76)

1. *Diluent*: Dissolve 85.0 gm. NaCl and 3.75 gm. Na-5,5-diethyl barbiturate in about 1400 ml. distilled water. Dissolve 5.75 gm. 5,5-diethyl barbituric acid in about 500 ml. hot distilled water. Mix the two solutions, cool to room temperature, add 5.0 ml. of a stock solution containing 1.00 M MgCl<sub>2</sub> and 0.30 M CaCl<sub>2</sub>. Add distilled water to exactly 2000 ml. (It is advisable to check concentration of MgCl<sub>2</sub> and CaCl<sub>2</sub> solutions by specific gravity and/or conductimetric titration with standard AgNO<sub>3</sub>.) Store in refrigerator.

*Alternative Procedure*: Dissolve 83.0 gm. NaCl and 10.19 gm. Na-5,5-diethyl barbiturate in about 1500 ml. distilled water. Add 31.2 ml. of exactly N/1 HCl, 5.0 ml. of a stock solution containing 1.00 M MgCl<sub>2</sub> and 0.30 M CaCl<sub>2</sub>, and enough water to make exactly 2000 ml.

For use, make precise five-fold dilution with distilled water in volumetric flask. Check pH (should be 7.5) and specific conductance (should be 0.00817 ohms<sup>-1</sup> cm<sup>-1</sup>). Discard unused portion of isotonic diluent at end of day.

It should be noted that most of the experiments reported from this laboratory

between 1949 and 1959 were made with a buffer mixture containing veronal and sodium bicarbonate. Recently it was found that the hemolytic activity of complement is sensitive to pH when Ca<sup>++</sup> and Mg<sup>++</sup> are present in optimal concentrations, and in view of the uncertainty regarding pH which the use of sodium bicarbonate introduces, it was decided to omit this salt.

Spontaneous lysis of red cells, as reflected in the cell blank, which is an essential part of every hemolytic titration, can be reduced by incorporation of 0.1% bovine serum albumin or gelatin in the buffer. The dry protein can be added to the buffer after dilution to isotonicity. Usually, gelatin is to be preferred because it is superior as a stabilizing agent and its cost is lower.

2. *Sheep Erythrocytes*: Sheep blood, drawn aseptically, is preserved at 2-5°C. in an equal volume of sterile, modified Alsever's solution (77), prepared as follows: 24.6 gm. glucose, 9.6 gm. sodium citrate (dihydrate), and 5.04 gm. NaCl are dissolved in 1200 ml. distilled water. Adjust to pH 6.1 with citric acid and sterilize by passage through ultrafine sintered glass filter. Sheep blood preserved in this manner is allowed to age for one week prior to use. After this stabilization period, the susceptibility of the erythrocytes to lysis by antibody and complement remains uniform for about two months, and a batch of blood may be used during this entire period, provided gross microbial contamination is avoided. It should be noted that the lytic susceptibility of red cells from different individual sheep may vary. Pooling of blood from several ani-

imals should be avoided. Do not bleed any one animal more than once every eight or ten weeks. Each bleeding may be as large as 500 ml.

For preparation of the standardized sheep cell suspension, a volume of blood sufficient for the day's work is withdrawn aseptically from stock and centrifuged. The plasma and buffy coat are carefully aspirated and the sedimented cells are washed three times with 5 to 10 volumes of the isotonic diluent. The second and third wash fluids should be colorless. The sedimented washed cells are evenly suspended in approximately 18 volumes of diluent to make an approximate 5% suspension. This suspension is filtered through a tiny wad of absorbent cotton in a funnel, and exactly 1.00 ml. of the filtered suspension is lysed with precisely 14.0 ml. of 0.1% aqueous solution of anhydrous sodium carbonate (the use of a 15 ml. volumetric flask is convenient). The optical density of the clear lysate is measured against a water blank in a Beckman spectrophotometer with a 1 cm. cuvette at a wave length of 541 mμ. An optical density (O.D.) of 0.700 corresponds approximately, in terms of the hemoglobin concentration, to  $1 \times 10^9$  erythrocytes per ml. of cell suspension, provided the sheep blood is derived from animals in healthy condition (57, 62).

The five per cent cell suspension will yield a lysate of O.D. slightly greater than the desired standardization value of  $0.700 \pm 0.005$ , and needs to be adjusted by dilution according to the following relation:

$$V_f = V_i \frac{\text{O.D.}}{0.700}$$

where O.D. represents the optical density of the lysate of the 5% suspension,  $V_i$  is an accurately measured aliquot of the approximate 5% erythrocyte suspension and  $V_f$  is the final volume to which it should be adjusted by addition of diluent. Check adjustment after making appropriate dilution. In case a photometer other than

Beckman DU is used, make sure O.D. varies linearly with hemoglobin concentration. If not, make calibration curve and use it for calculating adjustment.

After standardization in the manner just described, the cells are kept in a stoppered flask at refrigerator temperature until needed for titration.

**3. Preparation of Hemolytic Antibody and Sensitization of the Cells:** Hemolysin is made by inoculation of rabbits with boiled sheep erythrocyte stromata, which can be prepared as follows (67):

Collect 1 liter of sheep blood in 250 ml. of 3.8% sodium citrate (dihydrate), with continuous mixing. After filtration of the blood through cheese cloth to remove any clumps, sediment the cells by centrifugation in the cold and wash once with about one to two volumes of isotonic saline.

Acidify 10 liters of ice-cold distilled water by addition of four ml. of glacial acetic acid and add the packed red cells slowly with constant vigorous mixing. Continue stirring for about ten minutes. Let the mixture stand in the cold room overnight to permit the red cell stromata to settle. Next day, draw off as much of the supernatant fluid as possible. Transfer the stromata to at least six 250 ml. centrifuge bottles, centrifuge for fifteen minutes in the cold at 2000 rpm (International PR-1 or PR-2 centrifuge) and remove the supernatant fluid by suction. Wash the stromata four to six times with cold 0.001 M acetate buffer, pH 5. Use as much buffer as the centrifuge bottles will hold, and after each wash, centrifuge in the cold for fifteen minutes at 2000 rpm. (For large-scale preparations, washing with acetate buffer may be performed in 50 liter carboys; use two washes, each with 5 to 10 liters of acetate buffer for the stromata from one liter of blood, and let stromata settle overnight in the cold room.) Suspend stromata in an equal volume of cold 0.15 M NaCl and transfer to eight heavy-wall 100 ml. centrifuge bottles. Spin in an angle head for

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*Complement and Complement Fixation*

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twenty minutes at 4000 rpm and 0°C. Wash twice in cold 0.15 M NaCl with centrifugation at 4000 rpm in angle head. As the acetate is washed out, the stromata may settle less readily and, if so, the time of centrifugation should be increased. Suspend in 0.15 M NaCl to a volume of about 300-400 ml.

Transfer the suspension to an Erlenmeyer flask closed with cotton plug, add a magnetic stirring bar, and heat the stromata for one hour at 100°C. by immersion of the flask in a boiling water bath. Cool, place on magnetic stirring apparatus and stir until a smoothly dispersed suspension is obtained. Analyse a sample for nitrogen by micro-Kjeldahl analysis and then add sterile saline to make a suspension containing 1 mg. of nitrogen per ml. Add merthiolate to a final concentration of 1/10,000 (w/v).

Rabbits are immunized with this suspension by intravenous inoculation according to the following sequence: One injection of 0.1 ml., five injections of 1 ml., and five injections of 2 ml., each. The whole series of eleven injections covers a period of about two weeks; do not skip more than two consecutive days. Bleed the rabbits on the fourth and the fifth day following the last inoculation. Inactivate by heating at 56°C. for thirty minutes. Store at -20°C. For use, make a 1/100 stock dilution, which can be stored indefinitely at -20°C.

In order to determine the dilution of antiserum needed for optimal sensitization, 5.0 ml. portions of a series of antiserum dilutions (1/400, 1/800, 1/1600, 1/3200, 1/6400) are added to 5.0 ml. portions of standardized suspension of sheep cells (10<sup>9</sup> cells per ml.) in 40 ml. centrifuge tubes with constant swirling of the contents. One ml. portions of each of these cell suspensions are distributed in a series of 40 ml. centrifuge tubes, 5.5 ml. of isotonic diluent are added to each, followed by 1.0 ml. of an appropriate C' dilution. The C' dilution is chosen so as to yield approxi-

mately 50% to 70% hemolysis (for optimally sensitized cells); a 1/200 dilution of fresh guinea pig serum should accomplish this. The tubes are then incubated at 37°C. for ninety minutes with occasional agitation in order to maintain the cells in uniform suspension. At the end of this period they are centrifuged and the degree of lysis in each tube is estimated by photometric analysis of the supernatant fluid as in a complement titration. The titration with each cell population should be performed in duplicate, and for each of the cell suspensions duplicate cell blanks should be set up. In addition, for each of the cell suspensions a pair of tubes yielding complete lysis should be included. These "completes" are set up with 1.0 ml. of cell suspension and 6.5 ml. of the 1/200 dilution of complement. A few ml. of this complement dilution should be saved so that its optical density can be measured. This reading of the "complement color" is applied on a proportional basis as a correction to the O.D. readings of the supernatant fluids from the analyses proper.

On the basis of the titrations of hemolytic antibody, as described above, an appropriate dilution is prepared for optimal sensitization (cf. Fig. 43), and this dilution is slowly pipetted into an equal volume of standardized cell suspension with constant swirling of the contents (Do not reverse order of addition.) The flask is stoppered and placed in a refrigerator until needed. Sensitized cells are prepared daily as required.

**4. Complement:** Pooled guinea pig blood is allowed to clot for one to two hours at room temperature. The serum is separated from the clot, frozen immediately and stored at -40°C. or lower, in an electric deep freeze. If a dry-ice storage box is used, the serum should be kept in sealed glass ampoules.

For removal of natural hemolytic antibody, 100 ml. ice-cold guinea pig serum

are mixed with 3 ml. of packed sheep erythrocytes, which have been washed with saline. Keep at 0°C. for ten minutes, centrifuge at 0°C. and draw off the serum. Repeat this absorption once or twice. The quantities of serum desired for one day's work are then distributed in chilled test tubes; stopper, freeze promptly and store at -40°C., or lower. Under these conditions of storage, hemolytic complement activity stays constant for at least six months. At -20°C. the serum can be stored for one month without appreciable loss of activity.

For use, the contents of one or several tubes are thawed, and as soon as the ice has melted, the tubes are placed in ice-water and kept cold until needed. Dilutions should be made with ice-cold diluent.

Fresh-frozen, pooled guinea pig serum is available commercially in bulk quantities. Absorb with packed, washed sheep erythrocytes, distribute in tubes, and store at -40°C., or lower, as described above.

If facilities are not available for storage at low temperature, lyophilized guinea pig serum, which is available commercially, can be used, but its hemolytic titer is somewhat lower than that of the fresh-frozen serum.

#### Titration Procedure

The following sequence of reagents is recommended because it facilitates requisite mixing:

- (1) 1.0 ml. sensitized cells
- (2) The amount of isotonic veronal buffer required to make the final reaction volume of 7.5 ml.
- (3) The desired volume of an appropriate dilution of complement added from accurately calibrated pipettes, held to the inside wall of the tube above the surface of the liquid. The reaction is performed in wide-mouth 40 or 50 ml. centrifuge tubes which permit mixing

of the cells by rotary motion during addition of complement. The tubes are stoppered or capped to prevent evaporation of water during incubation.

If experimental determination of the hemolytic response curve is desired, a series of closely spaced dilutions (approximately 1.1 or 1.2-fold steps) of complement is prepared with accurately calibrated pipettes and volumetric flasks. In the reaction system described here, 1.0 ml. of a 1/200 dilution of guinea pig serum will produce a degree of lysis ranging between 40% and 70%, and hence, 1.0 ml. portions of a series of spaced dilutions from about 1/130 to about 1/300, or 1/350, should yield a series of points covering the entire range of the response curve. The preparation of such a series of dilutions with accurately calibrated pipettes is quite laborious, and therefore, a simpler procedure may be used in which an accurate 1/800 dilution of guinea pig complement is prepared and 2.50, 3.00, 3.50, 4.00, 5.00 and 6.00 ml. portions of this dilution are added with accurately calibrated pipettes to a series of reaction tubes. (Each reaction mixture must be made to the same total reaction volume, i.e., 7.5 ml., because of the "volume effect" in the hemolytic action of complement).

It is advisable to set up the reaction mixtures in a bath of ice-water in order to retard the action of complement until all tubes are prepared. The entire set is then placed in the water bath at 37°C  $\pm$  0.2° for sixty to ninety minutes with occasional mixing of the contents to keep the cells in uniform suspension. Each set of titrations should include duplicate cell blanks, as well as duplicate "completes" for which 1 ml. of a 1/40 dilution of guinea pig complement, or equivalent, can be used.

At the end of the incubation period, the tubes are centrifuged to remove unlysed cells, and the clear supernatant fluids are analyzed photometrically for oxy-

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hemoglobin at a wave length of 541 m $\mu$  in the Beckman DU Spectrophotometer. In addition, an optical density reading is taken of a low dilution of the guinea pig complement (e.g., 1/40) in order to obtain the correction for complement color. The O.D. readings of the supernatant fluids from all of the tubes, including the "completes" are corrected by subtraction of the O.D. values of the cell blanks, which should not exceed 1 or 2% lysis, as well as by subtraction of the appropriate complement color values. In addition, it is advisable to apply corrections, positive or negative, for light absorption, if any, by the spectrophotometric cuvettes when filled with water. It is not uncommon to find that cuvettes differ in such blank readings by O.D. values of 0.002 or 0.003. The spectrophotometric cuvettes should be checked also for uniformity of light path, and any appreciable deviation should be corrected factorially. After application of all of these corrections, the fraction of cells lysed in each tube is calculated by dividing its corrected O.D. value by the corrected O.D. of the complete.

It is convenient to represent results graphically by plotting  $\log \left( \frac{y}{1-y} \right)$  against  $\log x$ , where  $x$  is the relative concentration of complement. The use of 2 x 3 cycle log-log graph paper is recommended. (Alternatively, the probit function can be used for evaluation of results, ref. 78). Fit the best line to the experimental points and read the 50% lytic dose of complement from the graph. The complement titer is defined as the number of C'H<sub>50</sub> contained in 1 ml. of undiluted serum. For example, if 4.0 ml. of a 1/800 dilution of guinea pig

serum are required for 50% hemolysis, the titer equals 200 C'H<sub>50</sub> per ml.

In addition to evaluating the results of a titration in terms of C'H<sub>50</sub>, it is recommended that the value of  $1/n$  be checked by measuring the slope of the line representing the logarithmic form of the von Krogh equation. Under the experimental conditions described here,  $1/n$  should equal  $0.2 \pm 0.02$ . Serious deviation of  $1/n$  from the value 0.2 may indicate experimental error.

Sometimes it is not practicable to perform hemolytic analyses at several points within the range of partial lysis. If one point is available between 10 and 90% hemolysis, or better between 20 and 80%, the 50% unit can be calculated from the von Krogh equation by assuming that the value 0.2 for the exponent  $1/n$  is applicable. The need for calculation can be avoided by use of the conversion factors given in Table 3. It should be emphasized that titrations based on a single point are valid only if there is sound basis for assuming a value of 0.2 for  $1/n$ .

There are occasions when it is desirable to scale down the complement titration procedure. The technic described here is recommended from the standpoint of accuracy of pipetting and photometric analysis, but if desired, titrations can be performed in small reaction systems. Results will be the same, provided all reagents are scaled down proportionately. For example, with a sample of guinea pig complement containing 200 C'H<sub>50</sub> per ml., 0.2 ml. of a 1/200 dilution added to 10<sup>8</sup> sensitized cells in a total reaction volume of 1.5 ml. will yield 50% lysis.

#### PROCEDURE FOR TITRATION OF HEMOLYTIC ANTIBODY

The unit of hemolytic antibody, AbH<sub>50</sub>, is defined as that amount of antiserum in milliliters which in the presence of 12 fifty % units of complement (12 C'H<sub>50</sub>), optimal Ca<sup>++</sup> and Mg<sup>++</sup>, and at an ionic

strength of 0.147 will lyse 500 million red blood cells out of a total of 1 billion cells in exactly fifteen minutes in a total reaction volume of 5.0 ml. at a temperature of 37°C. (There are situations in which it

may be desirable to change the amount of complement; this will affect the titer of hemolysis.)

### Reagents

1. Standardized erythrocyte suspension containing  $5 \times 10^8$  cells per ml.
2. A series of accurately prepared dilutions of the hemolytic antiserum, spaced by a factor of approximately 1.2.
3. A dilution of pooled guinea pig complement containing 6 C'H<sub>50</sub> per ml., usually about 1/30 or 1/40 is required. Natural hemolysin of the guinea pig serum must be absorbed with sheep erythrocytes (cf. complement titration).
4. Ice-cold isotonic citrate-saline solution: 1 part 0.075 M aqueous sodium citrate (dihydrate) mixed with 4 parts of 0.15 M aqueous NaCl. This solution serves to stop the lytic reaction.

### Procedure

Measure out a series of 2.0 ml. portions of the cell suspension into 40 or 50 ml. centrifuge tubes. Add 1.0 ml. each of the appropriate dilutions of hemolytic antiserum slowly and with constant mixing, so as to ensure uniform distribution of the hemolytic antibody upon the cells. Incubate fifteen minutes at 37°C. Add 2.0 ml. of the diluted guinea pig complement (12 C'H<sub>50</sub>) and incubate at 37°C. with occasional mixing to maintain the cells in uniform suspension. After exactly fifteen minutes, add 10.0 ml. of ice-cold citrate-saline solution, mix, centrifuge, remove the clear supernatant fluid and determine

oxyhemoglobin photometrically as in complement titration.

The period of fifteen minutes' incubation for the lytic reaction must be timed precisely. This can be done by spacing the addition of complement to individual reaction tubes at intervals of exactly one-half or one minute. At the end of the fifteen minute reaction period, the citrate-saline solution is added to the tubes in the same sequence at intervals of exactly one-half or one minute.

A more convenient, though less accurate, scaled-down procedure is as follows: Use 0.5 ml. portions of red cell suspension, 0.5 ml. of antiserum dilution, 0.25 ml. of a dilution of guinea pig complement containing 12 C'H<sub>50</sub> per ml. (ca. 1/15 or 1/20) and 2.5 ml. of citrate saline. In essence, this modified procedure represents an analysis with  $\frac{3}{4}$  the quantities of all the reagents, except for two changes; one involves doubling the volume of antiserum for the sake of greater precision of measurement. The other change involves a corresponding decrease in the volume of complement from 0.5 to 0.25 ml., in order to maintain the total reaction volume at the proper level (1.25 ml.). Correspondingly, the dilution of the guinea pig complement is changed by a factor of one-half. Since twice the volume of antiserum is used, its dilutions should be double those employed in the large scale titration.

Use the von Krogh equation (or probit function) to evaluate data, as in the titration of complement. In the large scale assay, the titer of the hemolytic antiserum equals the reciprocal of the dilution yielding 50% hemolysis. In the small scale titration, the reciprocal of the dilution yielding 50% lysis is divided by two in order to obtain the titer.

### INHIBITION ASSAY

Assays of an antigen or hapten which combines with hemolytic antibody may be performed by mixing suitable quantities

of the antigen or hapten with an arbitrarily chosen amount of hemolytic antibody, e.g., 10, 100 or 1,000 AbH<sub>50</sub>. These mixtures

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## Complement and Complement Fixation

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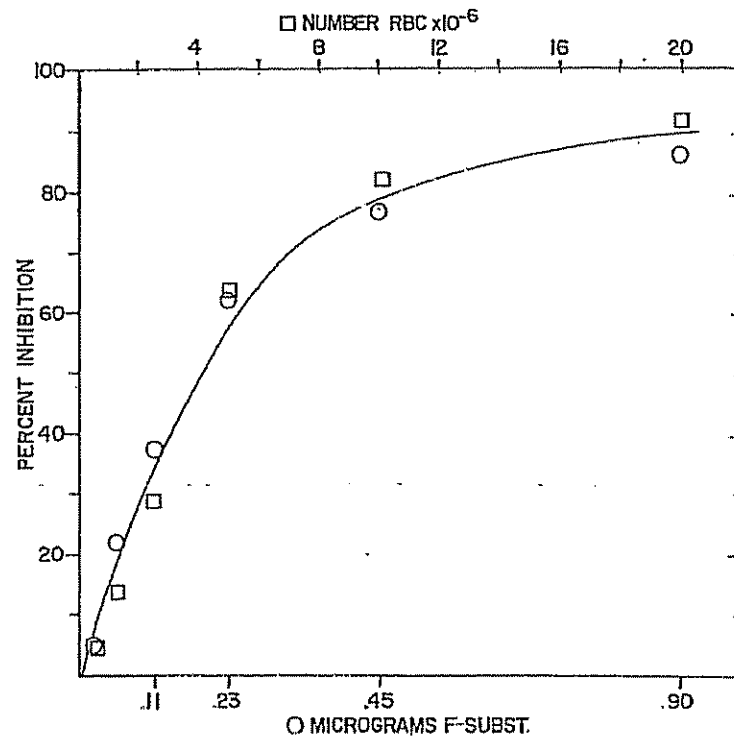


FIG. 44. Comparison of the reactivity of Forssman hapten and sheep erythrocytes with rabbit antiserum to boiled sheep red cell stromata. From (67)

are allowed to react for one-half hour at room temperature, and then appropriate dilutions are made which are titrated for residual activity of hemolytic antibody (in concentrated systems a precipitate may form on interaction of the antigen with hemolytic antibody, and this should be removed by centrifugation before the dilutions are prepared). It is convenient to represent the results as a plot of percentage inhibition vs. the quantity of inhibiting antigen or hapten (cf. Fig. 44). With antisera of very low dissociation tendency, the quantity of antigen required for a given percentage of inhibition, e.g., 50%, is directly proportional to the amount of hemolytic antibody in the test. With antisera of appreciable dissociation tendency, the efficiency of inhibition increases with concentration.

A rapid inhibition assay may be per-

formed with ordinary serological pipettes in 13 x 100 mm. pyrex test tubes, as follows: Mix 1.0 ml. portions containing varying amounts of antigen with 1.0 ml. portions of antiserum containing 7 AbH<sub>50</sub> and let react one-half hour at room temperature. To three 0.5 ml. portions of red cells, add, with mixing, 0.125 ml., 0.25 ml. and 0.5 ml. of inhibition mixture; equalize volume by addition of 0.375 ml. and 0.25 ml. diluent to the first two tubes, respectively. In addition, set up cell blank (cells plus diluent), "complete" (cells plus excess hemolytic antibody), and control (0.5 ml. of 1/7 dilution of reaction mixture containing no inhibiting antigen). Incubate at 37°C. for fifteen minutes. Lysis is initiated by adding 0.25 ml. of guinea pig complement dilution containing 12 C'H<sub>50</sub> per ml. After exactly fifteen minutes, add 2.5 ml. of ice-cold isotonic citrate-saline

solution to stop the lytic reaction. Centrifuge and determine hemoglobin in supernatant fluid. Apply corrections, calculate residual units of antibody ( $AbH_{10}$ ), and subtract these values from control to obtain units of antibody which were bound. Record results by plotting percentage inhibition against amount of inhibiting antigen.

### Explanation

With 7 units of antibody in the test, a control containing 1.0 ml. of antiserum, 1.0 ml. of diluent and no inhibiting antigen, would require a dilution of 1/7 to yield 50% hemolysis. In mixtures containing inhibiting antigen, a dilution less than seven-fold would be necessary, the magnitude of the dilution factor depending on the degree of inhibition. Thus, if dilutions of 1/4, 1/2 and 1/1 (undiluted) are tested, the entire range from zero inhibition to complete inhibition can be covered. Instead of preparing these dilutions, 0.125 ml., 0.25 ml. and 0.5 ml. of reaction mixture are used and diluent is added to maintain constant reaction volume. This is equivalent to 0.5 ml. of 1/4, 1/2 and 1/1 dilutions, respectively. The control mixture of antiserum and diluent should be diluted 1/7.

### QUANTITATIVE DETERMINATION OF COMBINING N OF COMPLEMENT (C' N)

The capacity of complement to combine with specific precipitates affords a means of measuring the serum N associated with the combining components. The method, originated by Heidelberger (33-37), involves determination of the difference in N content of specific precipitates formed in the presence and absence of active complement. Since the actual mass of complement combining components is quite small, it is necessary to use at least 2

### Precautions

(1) All glassware should be thoroughly clean and dry. It is especially important to check absence of substances which hemolyze red cells. Sulfuric acid-dichromate cleaning solution is satisfactory. Calgonite, or other detergents, or green soap may also be used. Copious rinsing is essential.

(2) All reagents should be stored in ice-water during use. Complement dilutions and sensitized cells are good only for one day.

(3) Care should be taken to avoid gross bacterial contamination of the reagents, since this may produce inhibitory effects. For this reason the veronal buffer is made up and stored in concentrated form. The 1/5 dilution to isotonicity should be made fresh each day and isotonic buffer which is unused should be discarded at the end of the day.

Preservation of erythrocytes under aseptic conditions is especially important in studies involving titration of hemolytic antibody, since microbial antigens, if produced in the blood, may be adsorbed to the red cells.

(4) Care should be exercised to avoid variations of ionic strength exceeding  $\pm 1\frac{1}{2}\%$ .

(5) In the sensitization step the antiserum must be added to the cells with efficient mixing. Do not reverse order of addition.

milliliters of active complement for a determination. Numerous previous attempts were unsuccessful due to failure to recognize this.

Quantitative determinations of C'N on guinea pig (34), human (37) and bovine (38) sera, have shown that the C'N per ml. of serum found experimentally depends on the amount of complement used for analysis, being progressively smaller when larger amounts of complement are em-

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played for analysis, despite complete removal of complement as determined by absence of hemolytic activity in the supernatant fluid. Figure 45 shows this apparent solubility effect in the case of guinea pig C'. By extrapolation to zero volume of C' it was estimated that guinea pig serum contains about 0.04 to 0.06 mg. C/N per ml. (34), while human serum gave values ranging from 0.03 to 0.05 mg. C/N per ml. (37). Similar results were obtained in the case of bovine sera (38).

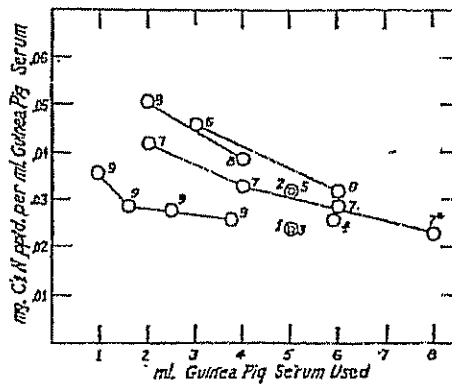


FIG. 45. Amount of complement combining component nitrogen (C/N) precipitated as a function of the volume of guinea pig serum used.

The numbers at each point refer to the experiment. 7\* = combined serum and S III blank supernatants. From (34).

The assumption that at least part of the N carried down by specific precipitates from active sera constitutes complement protein is supported by several obser-

vations. As stated above, hemolytic complement activity is removed by absorption with specific precipitates, that is, after removal of the specific precipitate the supernatant fluid no longer lyses sensitized sheep erythrocytes. When very small amounts of washed specific precipitate are added to a large volume of complement, partial removal of hemolytic activity, as well as of combining N, result. With varying amounts of specific precipitate, uptake of nitrogen by the specific precipitate and disappearance of hemolytic activity from the supernatant fluid run roughly parallel (36).

The fact that no N was taken up from complement inactivated for fifty minutes at 56°C. also supports this assumption. Furthermore, with pneumococcus specific polysaccharide and the corresponding horse antibody, which does not fix complement effectively (see below), as judged by hemolytic activity, no N uptake was observed (34).

By use of EDTA, Laporte (79) as well as Becker (80) have dissociated C'1 from sensitized red cells treated with complement. This proves C'1 enters into physical union with antigen-antibody aggregates, and that at least part of the C' combining N represents C'1.

However, since rheumatoid factor combines with antigen-antibody aggregates (17), and since conglutinin combines with antigen-antibody-complement complexes (18), measurements of complement combining N must be interpreted with caution.

#### PROCEDURE FOR QUANTITATIVE ESTIMATION OF COMPLEMENT COMBINING N

The procedure may be illustrated with the aid of the protocol of a typical experiment shown in Table 5. Before use, the guinea pig serum was neutralized to about pH 7 and centrifuged thoroughly. One-half was inactivated at 56° C. for fifty minutes, instead of thirty minutes, since it was found (34) that the standard thirty

minute period did not always suffice to reduce to a minimum the amount of nitrogen taken up by specific precipitates from inactivated complement (iC'). Both the fresh guinea pig serum and the inactivated serum were centrifuged in the cold for several hours before use. Diluted, neutralized and inactivated antipneumococcus

TABLE 5  
Quantitative Estimation of Combining N of Complement

| No. of tubes.....                    | 1     | 1     | 2   | 1     | 1     | 3     | 3      | 3      |
|--------------------------------------|-------|-------|-----|-------|-------|-------|--------|--------|
| C', ml.....                          | 4.0   | 4.0   |     | 3.0   | 3.0   |       | 5.0    | 5.0    |
| iC', ml.....                         |       |       |     | 0.6   | 0.6   | 1.0   | 1.0    | 1.0    |
| Serum dilution, ml.....              | 0.8   | 0.8   | 1.0 |       |       | 1.0   | 1.0    | 1.0    |
| S III dilution, ml.....              |       |       |     |       |       | 0.584 | 0.634  | 0.740  |
| N precipitated, mg.....              | 0.012 | 0.016 | 0   | 0.018 | 0.020 | 0.594 | 0.624  | 0.738  |
|                                      |       |       |     |       |       | 0.586 | 0.636  | 0.736  |
| Mean                                 | 0.014 |       |     | 0.019 |       | 0.588 | 0.632  | 0.728  |
| Subtraction of blank.....            |       |       |     |       |       | 0     | 0.032* | 0.018† |
| Specific N pptd., mg.....            |       |       |     |       |       | 0.588 | 0.600  | 0.720  |
| Subtraction of iC' series value..... |       |       |     |       |       |       |        | 0.600  |
| C'N pptd., mg.....                   |       |       |     |       |       |       |        | 0.12   |

Hemolytic units left in C' series supernatants, 10 per 5 ml. C' taken.

\*0.019 x 5/3.

†0.014 x 5/4.

From (34).

Type III rabbit serum containing 1.0 mg. antibody N per ml. of dilution\* and a solution of homologous specific polysaccharide containing 0.04 mg. per ml. were mixed in equal proportions. The concentrations of antibody and antigen were chosen to leave antibody in excess so that a finely divided precipitate would be formed which could be washed more efficiently than the gelatinous discs obtained in the equivalence zone. Triplicate sets of analyses were performed with active complement (C'), heat-inactivated complement (iC') and with saline as diluent of the immune system. In addition, blanks of C' and iC' were set up with immune serum alone, and with polysaccharide alone. A blank on the immune serum alone was also included. C' and iC' were pipetted into the tubes first, followed by the antiserum. After mixing, the polysaccharide solution was added, the contents of each tube were mixed by twirling (I, 2) and kept at room temperature for one hour, or longer if

\*Sera of high antibody content (3 to 7 mg. antibody N per ml.) were used so that analyses could be made at dilutions which were not anticomplementary.

aggregation did not occur within one hour in the tubes containing active complement.

The tubes were then centrifuged in the cold, the precipitates washed three times, and analyzed for N as described for the quantitative precipitin reaction (I, 2) (81). All supernatants were again centrifuged as in the quantitative agglutinin procedure (I, 3) (82) to insure the greatest possible accuracy, since the experiment involved measurement of a small difference between two quantities of specific precipitate.

Hemolytic activity in the supernatant fluids from the tubes which had contained active complement was computed from the results on the largest non-anticomplementary volume which could be employed. Complete removal of hemolytic activity was obtained with quantities of specific precipitate about five times as large as the amount of C'N present, as in the experiment shown in Table 5.

For calculation, the nitrogen obtained in the iC' blank tubes (columns 5 and 6, Table 5) was averaged and subtracted from the specific precipitate N in the iC' series (column 8) while the average of the C' blanks (columns 2 and 3) was deducted

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from the specific precipitate C'N (column 9). Finally, the resulting net values were subtracted (0.720 minus 0.600) yielding the C'N value of 0.12 mg. This is equivalent

to 0.024 mg. of C'N per ml. of guinea pig serum since 5 ml. of serum were used in the analysis. (Cf. Fig. 45, Experiment No. 1).

### COMPONENTS OF COMPLEMENT

Complement is not a single serum constituent, but comprises several substances which act sequentially. At present five distinct factors, C'1, C'2, C'3a, C'3b and C'4 are recognized. The first and second components (C'1 and C'2) were discovered in 1907 by Ferrata (83), who separated guinea pig serum by dialysis against water into an insoluble and a soluble fraction which were subsequently termed midpiece and endpiece, respectively. The insoluble fraction, when dissolved in physiological saline, was devoid of hemolytic complement activity. The soluble fraction was also found to be inactive, but when the two were combined, activity was restored. Since then it has been shown that guinea pig complement can be split into these two components by a variety of procedures, leading to precipitation of euglobulin, such as by dilution with several volumes of water acidified to about pH 5, or by dialysis against very dilute buffer at about pH 5 to 6.

When complement is treated with cobra venom (84), yeast (85), or zymosan (86), an insoluble carbohydrate from yeast, its activity is destroyed because a factor other than C'1 or C'2 is inactivated or removed. This so-called third component of complement, C'3, is relatively heat-stable in contrast to C'1 and C'2, which are comparatively susceptible to heat. Pillemer *et al.* (87) have shown that removal of C'3 by zymosan is a two-step reaction. First, zymosan combines with a serum constituent which Pillemer called properdin, and the resulting complex then inactivates C'3 selectively. This concept has been challenged by Nelson (88) who proposed that zymosan combines with anti-zymosan normally present in blood serum to form an antigen-antibody complex which then

reacts with C'1, C'4 and C'2, yielding zymosan antibody-C'1, C'4, C'2. This product, which corresponds to Pillemer's zymosan-properdin complex, then fixes C'3. Thus, Nelson considers the inactivation of C'3 by zymosan as a complement fixation reaction in which the removal of C'3 is relatively greater than the fixation of C'1, C'4 and C'2, due to the choice of experimental conditions (For further discussion, see properdin section.)

DaCosta Cruz and Penna (89) claimed that C'3 is composed of two components, but their work did not receive much attention in the English literature until Rapp (90), as well as Amiraian *et al.* (91) showed that C'3 comprises two factors now designated C'3a and C'3b. This will be taken up later in detail; for the present discussion, C'3 will be treated as a single entity.

(Recent unpublished experiments by Rapp indicate that C'3 comprises three distinct factors.)

Still another heat-stable factor, the fourth component, C'4, is recognized by the fact that hemolysis can no longer occur after its destruction by ammonia (92) or primary amines (93). C'4 is also inactivated when guinea pig serum is shaken with ether or chloroform (94).

The complement components described so far have been studied extensively and their existence as distinct factors has been generally accepted. Claims have been made for the existence of several other components (94-98), but these will not be dealt with in this presentation, since their existence is controversial.

When complement is fractionated by dialysis or by precipitation of the euglobulin, C'1 and C'2 are the components which are separated from one another.

C'3 and C'4 are distributed in both fractions. Most of the C'3 activity remains with C'1 in the insoluble fraction, while the bulk of C'4 is found with C'2 in the supernatant fluid.

On heating at 56°C., complement loses activity within a few minutes because of the destruction of C'1 and C'2. C'3 and C'4 are more resistant, but if heating is continued for thirty to forty minutes, most of the C'3 and C'4 activity is also lost.

The evidence for the individuality of C'1, C'2, C'3 and C'4 may be summarized as follows (cf. Fig. 46):

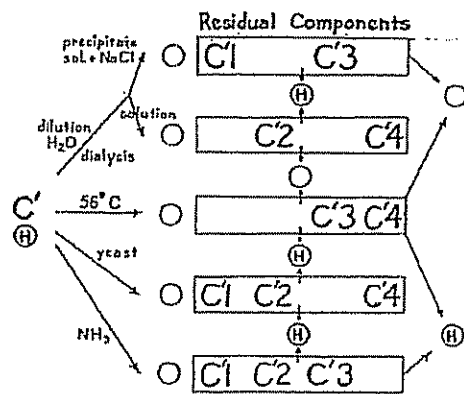


FIG. 46: Interrelations of Complement and its Components. Combinations giving hemolysis are denoted by an H.

(1) The soluble (E) and insoluble (M) fractions obtained on dialysis or by dilution with water, are inactive alone, but activity is restored by reconstituting the mixture.

(2) Complement treated with yeast or zymosan (Z) and ammonia-treated complement (N) are inactive when tested separately, but the combination of Z + N is active.

(3) Complement heated at 56°C. for fifteen or twenty minutes (H) is inactive alone, but in combination with Z or N hemolysis is obtained. Hence, heated serum contains those components which are removed or destroyed by zymosan and by ammonia, namely, C'3 and C'4.

(4) Combinations of heated serum with either M or E are inactive. Hence, heated serum does not contain C'1 and C'2. It also follows that the components removed or destroyed by zymosan and ammonia, namely, C'3 and C'4, respectively, are distinct from C'1 and C'2, the factors which are separated when complement is split into M and E.

The insoluble (M) and soluble (E) fractions, zymosan-treated complement (Z) and ammonia treated complement (N), can be used as reagents in testing for those components which they lack. For example, if an unknown solution possesses no activity when used alone, but activates zymosan-treated complement (Z), it is presumed to furnish the C'3 which is lacking in Z. Thus, Z is used as a reagent for the detection of C'3. Similarly, N is used as a reagent for C'4, M is used for detection of C'2 and E serves for demonstration of C'1.

In this fashion, Hegedus and Greiner (99) titrated the four components in the sera of various animals. They made the assumption that the titer of whole C', or of a mixture of components, is a function of the component present in lowest titer, the so-called limiting component. To determine the titer of a component in an unknown, they used a reagent which does not contain the component to be titrated (as shown by inactivity when used alone), but which supplies an excess of the other three components, so that the component in question becomes the limiting component in the mixture of unknown and reagent. The titer of this mixture is taken as the titer of the component sought.

Bier *et al.* (100) proposed that the reagents for titration of C'1, C'2, C'3 and C'4 be designated R1, R2, R3 and R4, respectively. Hegedus and Greiner (99), as well as Ecker *et al.* (101), used endpiece (E) as R1, and midpiece (M) as R2, but E is deficient in C'3 and M lacks adequate C'4 (cf. Table 6). Therefore, Bier *et al.* (100) recommended reinforcement of E and M by addition of heated serum (H) to

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## Complement and Complement Fixation

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TABLE 6

Average Composition of Whole and Fractionated Complement and of Specifically Inactivated Complement\*

|                            | Whole C'<br>units/ml. | C'1<br>units/ml. | C'2<br>units/ml. | C'3<br>units/ml. | C'4<br>units/ml. |
|----------------------------|-----------------------|------------------|------------------|------------------|------------------|
| G.P. C'                    | 350                   | 2300             | 450              | 370              | 6000             |
| G.P. M, prep'd by dialysis |                       | 350              | 0                | 120              | 100              |
| G.P. M, prep'd by dilution |                       | not done         | 0                | 100              | not done         |
| G.P. E prep'd by dialysis  |                       | 0                | 140              | 60               | 3500             |
| G.P. E prep'd by dilution  |                       | 0                | 220              | 60               | 2100             |
| G.P. Z                     |                       | 1000             | 260              | 0                | 2000             |
| G.P. N                     |                       | 1200             | 300              | 100              | 0                |
| G.P. H†                    |                       | 0                | 0                | 210              | 500              |
| Hu. C'                     | 100                   | 3700             | 170              | 250              | 4000             |
| Hu. M prep'd by dialysis   |                       | 1000             | 0                | 90               | 350              |
| Hu. M prep'd by dilution   |                       | 1000             | 0                | 40               | 130              |
| Hu. E prep'd by dialysis   |                       | 0                | 110              | 35               | 2400             |
| Hu. E prep'd by dilution   |                       | 0                | 115              | 45               | 2400             |
| Hu. Z                      |                       | 2000             | 100              | 0                | 2000             |
| Hu. N                      |                       | 2000             | not done         | not done         | 0                |
| Hu. H†                     |                       | 0                | 0                | not done         | 600              |

\*Compiled from data in (100).

†Heated at 56° C for 20 min.

each of these fractions. Thus, R1 = E + H, and R2 = M + H. Since Z contains C'1, C'2 and C'4 and N has C'1, C'2 and C'3 presumably in adequate amounts, they can function as R3 and R4, respectively (100).

The adequacy of each reagent with respect to its component content is established by testing every reagent with every other one, as follows: (1) R1 and R2, (2) R1 and R3, (3) R1 and R4, (4) R2 and R3, (5) R2 and R4, and (6) R3 and R4. All of these mixtures should be hemolytically active, proving that each reagent actually contains the components it is designed to have. In addition, it is necessary to show that each reagent is inactive alone and that it is not anticomplementary (100).

Another assumption made by Hegedus and Greiner (99) is that the components of complement in the sera of different species are mutually substitutive, that is, a given component from one species can function if the lacking components are supplied by the serum from another species. While the effectiveness of substitution was at first denied in the case of human and guinea pig C' (101), the work of Bier and collaborators (100) showed that the four components of human and guinea pig

serum are mutually substitutive although the exchange may not always be accomplished easily. This conclusion was also reached (102) by the former workers.

In (100), the four components were titrated in whole human and guinea pig sera, in "midpiece" and "endpiece," in the specifically inactivated complements and in heated serum. Results, summarized in Table 6, show that M contains C'1, C'3 and a little C'4; E contains C'2, some C'3 and much C'4, Z contains C'1, C'2 and C'4, while N contains C'1, C'2 and C'3. Since most of the C'3 appears in M, while most of the C'4 is found in E, M and E have been represented in Figure 46 to contain C'1,3 and C'2,4, respectively.

Hegedus and Greiner (99) studied the components of cow, dog, horse, pig, rabbit, rat, sheep as well as guinea pig and human complement. They found some species such as the cow, horse and sheep to be lacking in C'2. Sheep complement lacked C'4 as well.

## Limitations

During the past ten years, it has become increasingly evident that titrations of the

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components with R1, R2, R3 and R4, are subject to much uncertainty, especially from a quantitative standpoint. Major difficulty arises from the postulate of Hegedus and Greiner (99) that the hemolytic activity of complement, or a mixture of its components, is limited by the component present in lowest titer, and the concomitant assumption that the concentrations of the other components do not influence the degree of lytic activity. The reagents R1, R2, R3 and R4 are supposed to supply all but one of the components in excess, so that the lacking component, for which a test sample is to be titrated becomes the limiting factor and thus controls the hemolytic activity.

In practice, it is difficult to make preparations of the reagents which meet these conditions in a satisfactory manner. The lower limit for use of a reagent is the least quantity which will produce full activity in combination with every other reagent. The upper limit is the largest quantity which is not hemolytic by itself. The gap between these limits which can be achieved is frequently not wide enough to permit the use of an adequate excess of reagent.

Heidelberger *et al.* (103), Silverstein (104), as well as Jonson (105), have shown that the hemolytic titers of the complement components are dependent upon the quantity of reagent used for titration. Therefore, the titers are as much a reflection of the properties of the reagent as

they represent a measure of the component in question. There are at least two possible causes for this difficulty: 1) The reagent may not be completely free of the component to be titrated. This may apply even though the reagent is used at a level which is sublytic with respect to its action on sensitized cells (cf. studies by Leon *et al.* (73, 106, 107). 2) The quantity of reagent used may not furnish an excess of its components. The "limiting component" concept will be discussed further in a later section.

The technique of component titration with R1, R2, R3 and R4 also is unsound because no attention is paid to the sequence of action of components, i.e., sensitized cells, test sample and reagent are thrown together in any sequence and manner the experimenter happens to choose, with no rhyme or reason. As a result, inhibitory effects or competitive actions, or mutual enhancement, may influence the outcome.

In recent years, efforts have been made to devise new methods for the detection and titration of the complement components involving the use of the intermediate products formed in the sequential action of the components. The development of these new procedures is still in its early stages, and therefore, detailed experimental directions will be given for the old methods based on R1, R2, R3 and R4, with the stipulation that the results may be subject to uncertainty.

#### PROCEDURE FOR TITRATION OF COMPLEMENT COMPONENTS WITH R1, R2, R3 and R4

##### Preparation of Reagents

**A. Midpiece (M) and Endpiece (E):** Fractionation of human and guinea pig complement may be carried out by dilution or dialysis following Ferrata (83), Liefmann (108) and later workers (101, 109). While the CO<sub>2</sub> dilution method (108) may be used for fractionating guinea pig serum, it is not satisfactory for human serum.

**1. Dilution Method:** To 10 volumes of chilled 0.02M KH<sub>2</sub>PO<sub>4</sub> solution, 1 volume of chilled guinea pig or human serum is added slowly, with constant mixing in the cold. After twenty to thirty minutes at 0°C., the precipitate is centrifuged off in the cold, separated as completely as possible from the supernatant fluid and, in the case of guinea pig C', washed with phosphate solution of pH 5.4 and ionic strength

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0.02. The precipitate from human C' need not be washed since human serum contains much less C'2 than does guinea pig serum. The precipitate, or M, is dissolved in saline and brought to pH 6.5 to 7 by cautious addition of a freshly prepared 0.1 *N* NaHCO<sub>3</sub> solution. It is made up to five times the original serum volume for further dilution as needed. The original supernatant fluid, or E, is made isotonic with 10% NaCl and adjusted to about pH 7.5 with 0.1 *N* NaHCO<sub>3</sub> as soon as possible, since E is unstable below pH 6.5 (112, 113). The final dilution is 1/12.

**2. Dialysis method (101):** Fifteen ml. of human serum in cellophane tubing are dialyzed in the cold for twenty-four hours on a mechanical stirrer (III, 43) against four liters of phosphate buffer of ionic strength 0.02 and pH 5.4. For larger amounts of serum more time should be allowed and the phosphate buffer should be changed once or twice. Bier and co-workers (100) shortened the dialysis period to six hours when 5 ml. portions of serum were used, to avoid deterioration of components unstable at acid pH. This was accomplished by increasing the dialyzing surface by placing a pyrex test tube inside the cellophane bag to force the serum into the resulting annular space, by frequent mixing and by changing the outside buffer solution every two hours.

After dialysis the contents of the cellophane bag are centrifuged in the cold, the supernatant fluid is separated and neutralized by careful addition of 0.2 ml. of 0.1 *N* NaOH per ml. of supernatant fluid. The solution is then made isotonic by addition of 10% NaCl solution.

The precipitate (M) is washed twice with cold phosphate buffer of ionic strength 0.02 and pH 5.4 and dissolved in saline. The final dilutions are 1/5 for M and 1/10 for E.

The phosphate buffer for dialysis and washing is prepared by adding 153 ml. of 0.5 *M* KH<sub>2</sub>PO<sub>4</sub> solution and 1.73 ml. *N*

KOH to sufficient distilled water to make four liters of solution.

**B. Complement Lacking C'3:** The third component is inactivated by treatment with "zymosan," an insoluble carbohydrate from the cell wall of yeast (86, 110, 111). (Isliker, ref. 111, has shown that zymosan has very low, though measurable solubility). Zymosan is composed mainly of carbohydrate as a glucose polymer, but contains small amounts of nitrogen, phosphorus, magnesium and ribonucleic acid (110,111). Active preparations of zymosan are available commercially.\*

Different preparations of zymosan may vary widely in their capacity to inactivate C'3. Preparations which are relatively effective against C'3 have been designated "Type A." The term "Type B" refers to preparations which react with "properdin," are not effective against C'3 and make a satisfactory RP reagent (see section on properdin). Other types have also been described (110). The differentiation of these types is not sharp. Mild hydrolysis of zymosan with acid (111), or treatment with trypsin (110,111) or phenol (111) enhances activity against C'3. Carbohydrates from other sources, e.g., an insoluble dextran from *Leuconostoc mesenteroides* (111), also inactivate C'3. McNall (114) recommends the use of inulin† for inactivation of C'3.

The method of C'3 inactivation by zymosan given here is that recommended in (93, 101): Boil 100 mg. of zymosan in 10 ml. of saline for one-half hour. Centrifuge, discard the supernatant fluid and suspend the sediment in 100 ml. of saline. This stock suspension which contains 1 mg. per ml. is stored in the cold. Mix well before use. It is important\* that the zymosan particles be finely dispersed.

Ecker (101) recommends the use of 1.35 mg. of zymosan for inactivation of 1 ml.

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†Matheson Company, East Rutherford, N. J.